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13. ABSTRACT ***(Maximum 200 Words)***

We have been developing conditionally replicative adenoviral agents (CRADs) for prostate cancer gene therapy. We have sought to improve these agents by increasing their infectivity for tumor targets and by enhancing the specificity of their replication exclusively within tumor targets.

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A. INTRODUCTION

Replicative adenoviral vectors (CRAD) represent a promising therapeutic approach which has been applied recently in context of cancer of the prostate. As for the non-replicative cancer gene therapy approaches, however, the efficacy of the replicative strategies is subservient to vector-mediated tumor transduction. In this regard, one of the attractive features of the CRAD approach is that it capitalizes on the unparallel efficiency of adenoviral vectors (Ad) in accomplishing *in vivo* transduction. Indeed, of all of the currently available vector approaches, Ad vectors possess the highest capacity to achieve *in situ* transduction of tumor. Despite this capacity, overall efficacy of Ad-based cancer gene therapy approaches remain limited by suboptimal vector efficiency. Of note, human trials carried out to date have demonstrated relatively inefficient gene transfer to tumor achieved by Ad vectors employed in *in vivo* delivery schemas. This has been understood to result from a relative paucity of the primary adenovirus receptor, coxsackie-adenovirus receptor (CAR), on tumor cells relative to their cell line counterparts. Indeed, a relative paucity of CAR has been shown to limit Ad vector efficacy in a number of tumor contexts, possibly representing a fundamental practical barrier to realizing the full benefit of this vector system for cancer gene therapy applications. On this basis, it has been proposed that gene delivery via "CAR-independent" pathways may be required to circumvent this key aspect of tumor biology. Especially noteworthy in this regard, has been the observation in the Onyx trials of transductional-barriers limiting overall efficacy of CRAD-based replicative approaches. Thus, it is clear that augmenting the gene transfer efficacy of Ad vectors is essential to deriving their full benefit in the context of the conceptually promising CRAD strategies. To this end, we have developed novel approaches to alter the tropism of Ad vectors such that CAR-independent gene transfer may be achieved. Such CAR-independent gene transfer allows dramatic augmentations of the gene transfer efficacy of the Ad vector, especially in context whereby CAR levels are limiting. We thus propose to develop such modifications in the context of CRAD to improve its infection efficacy for tumor cells. We hypothesize that such modifications will overcome biological limits of tumor noted in human clinical trials and thereby allow full realization of the potential benefits of the CRAD approach. The demonstration of this principle, in model systems, would allow a rapid translation of this strategy into the human clinical context for prostate cancer therapy.

B. BODY

We are endeavoring to improve CRAd efficacy by addressing two key predicates of anti-tumor efficacy – infection efficiency and replicative specificity. In the former regard, the relevance of tumor cell Ad resistance on the basis of CAR deficiency has been generally recognized. On this basis, our initial studies focused on methods to alter Ad tropism to achieve CAR-independent infection. In the initial funding period we pursued a strategy of genetic capsid modification via incorporation of the integrin-binding peptide RGD4C into the fiber HI loop. This maneuver allowed the achievement of CAR-independent infection with improvement in CRAd potency. Herein we have explored additional strategies to achieve this end. In this regard, we considered the exploitation of non-serotype 5 receptors for Ad infection of tumor cells. Whereas the majority of Ad, and CRAd, agents have been

based on type 5 Ad, we considered routing the virus via the receptor of Ad serotype 3. To achieve this end we first studied the expression of the serotype 3 receptor on tumor cell targets. Our studies showed that unlike the type 5 Ad primary receptor (CAR), the serotype 3 primary receptor was not uniformly under-expressed. Indeed, type 3 receptor was frequently expressed at significantly elevated levels compared to CAR. On this basis, we derived Ad vectors which were mosaic with respect to the fiber knob. Studies demonstrated that the mosaic Ad was capable of infecting cells via the targeted serotype 3 receptor. Further, target cell infection levels paralled the levels of serotype 3 receptor characterizing tumor cells. It was thus clear that the knob serotype chimera strategy allowed the achievement of the linked goals of CAR-independent infection with infectivity enhancement. These findings rationalized the development of CRAd agents which incorporated this genetic capsid modification. Such 3/5-fiber knob mosaic CRAds were derived and analyzed for oncolytic potency. As predicted, CRAd agents capable of infection via the serotype 3 pathway exhibited enhanced oncolytic potency. These studies have established an additional method to achieve improved CRAd efficacy and offer the potential for synergy with the previously defined HI loop modification approach (Appendix A & B).

To address the therapeutic index aspects of CRAd utility, we have sought to improve the replicative specificity of our derived CRAds. In this regard, CRAds of the "tsp" type employ tissue/tumor selective promoters to control key functional Ad genes. A key consideration in considering promoter utility is the marked hepatotropism of Ad. On this basis, ectopic Ad localizes in the main (> 95%) at the liver. Mitigation of potential CRAd-based toxicity thus involves restricting the replication of CRAd replication of that portion of the agent which escapes tumor localization. To achieve this end, an ideal promoter would exhibit a "liver off/tumor on" phenotype. In the first period we have explored the utility of the promoters derived from the cycloxygenase-2 (cox-2) gene and the midkine (Mk) gene. We have endeavored studies which demonstrate that cox-2 exhibits a high level of inductivity in prostate cancer targets (Appendix C). In both instances CRAd agents have been derived and are currently being evaluated in animal model systems (Appendix D & E). In addition, we have identified additional promoter agents (tsps) which manifest the liver off phenotype and thus represent candidate prostate cancer tsps. These include promoters of the VEGF, GRP, SLPI and EpCAM genes. These promoters are currently being evaluated in prostate cancer targets as well as being configured into CRAd agents.

We have thus addressed, in the second funding period, two key predicates of CRAd physiology/efficacy – replicative specificity and infectious efficiency (Appendix F). Studies described herein have allowed the derivation of a class of "infectivity enhanced" CRAds based on at least two distinct genetic capsid modifications – fiber HI loop ligands and fiber knob serotype chimerism. In addition, our studies have identified a range of promoter elements compatible with the desired goal of tumor selective replication. Further studies will define the precise configuration of infectivity enhancement methods and replicative specificity promoter of optimal relevance for prostate cancer targets.

C. KEY ACCOMPLISHMENTS

- We have shown that the receptor of Ad serotype 3 can be exploited to achieve infectivity enhancement of tumor targets.
- We have shown that CRAd agents which achieve infection via the receptor of Ad serotype 3 can achieve enhanced oncolytic potency.
- We have identified additional promoter elements with potential relevance to the derivation of prostate cancer CRAds.
- These findings have established the basis of constructing an optimized CRAd agent for prostate cancer which embodies the desired properties of enhanced infectivity and replicative specificity.

D. REPORTABLE OUTCOMES

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E. PRESENTATIONS

Vector Targeting Strategies for Therapeutic Gene Delivery, Cold Spring Harbor Winter Biotechnology Conference, Co-Organizer, Cold Spring Harbor, New York, NY. March 15-18, 2001

American Association for Cancer Research 92nd Annual Meeting, Experimental Gene Therapy, New Orleans, LA. March 24-28, 2001

Second Annual CaP CURE Gene Therapy Meeting, Johns Hopkins University, Baltimore, MD. April 6-7, 2001

F. PATENTS

None

E. CONCLUSION

Our findings have established key facets of CRAD biology which have direct implications on their design/utility. Specifically, infectivity-enhancement maneuvers substantially improve the potency of these agents and enhance their anti-tumor capacity. In addition, we have defined novel regulatory elements relevant to prostate cancer CRAD design. These elements exhibit an ideal inductivity/specificity profile for the current application. In the aggregate, these advancements will allow the rapid derivation of a CRAD agent for prostate cancer with optimized biologic properties predicating anti-tumor efficacy.

The present studies suggest extend conclusions of the first funding period by the identification of additional methods for infectivity enhancement and additional promoters for achievement of replicative specificity. Derivative CRAd agents will thus embody both of these features as best suited for application of the context of cancer of the prostate.

APPENDIX A

Targeting Adenovirus to the Serotype 3 Receptor Increases Gene Transfer Efficiency to Ovarian Cancer Cells[1]

Anna Kanerva, Galina V. Mikheeva, Victor Krasnykh, Candace J. Coolidge, John T. Lam, Parameshwar J. Mahasreshti, Shannon D. Barker, Michael Straughn, Mack N. Barnes, Ronald D. Alvarez, Akseli Hemminki, and David T. Curiel[2]

Division of Human Gene Therapy, Departments of Medicine, Pathology, and Surgery and the Gene Therapy Center [A. K., V. K., C. J. C., J. T. L., P. J. M., S. D. B., A. H., D. T. C.], and Division of Gynecology Oncology, Department of Obstetrics and Gynecology [M. S., M. N. B., R. D. A.], University of Alabama at Birmingham, Birmingham, Alabama 35294-3300, and VectorLogics, Inc., Birmingham, Alabama 35294 [G. V. M., V. K.]

ABSTRACT

Gene delivery efficiency in clinical cancer gene therapy trials with recombinant adenoviruses (Ads) based on serotype 5 (Ad5) has been limited partly because of variable expression of the primary Ad5 receptor, the coxsackie and adenovirus receptor (CAR), on human primary cancer cells. As a means of circumventing CAR deficiency, Ad vectors have been retargeted by creating chimeric fibers possessing knob domains of alternate Ad serotypes. In this study, we have constructed an Ad5-based vector, Ad5/3luc1, with a chimeric fiber protein featuring a knob domain derived from Ad3. This virus is retargeted to the Ad3 receptor and, therefore, has different tissue tropism. A novel knob binding assay was used to measure expression of CAR and the Ad3 receptor. Further, to evaluate the correlation of receptor expression and infectivity by Ad, a panel of ovarian cancer cell lines and purified primary cancer cells were infected with Ad5luc1 and Ad5/3luc1 at 50, 200, and 1000 viral particles/cell. Our results confirm that Ad5/3luc1 is retargeted to the Ad3 receptor. Furthermore, the Ad3 receptor is present at higher levels than CAR on ovarian adenocarcinoma cells. Also, the amount of binding to primary receptor appears to be the major factor determining the efficiency of transgene expression. The Ad5/3 chimera displays enhanced infectivity for ovarian cancer cell lines and purified primary tumor cells, which could translate into increased efficacy in clinical trials.

INTRODUCTION

Epithelial ovarian cancer is the leading cause of gynecological cancer mortality in the United States with an estimated 23,400 new cases and 13,900 deaths in 2001. Most cases of ovarian cancer are diagnosed at an advanced stage, resulting in a 5-year overall survival of <30%, despite aggressive surgical debulking and chemotherapy (1). Thus, novel strategies for treatment of advanced stage ovarian cancer resistant to traditional therapeutic modalities are needed.

Gene therapy represents a promising treatment alternative that has recently displayed some clinical utility (2). In the context of ovarian cancer, Ads[3] have shown promise *in vitro* and in animal models (3). In most cases Ad vectors have been based on serotype 5, because of its capability of mediating high levels of transgene expression, its ability to transduce dividing and nondividing cells, and its broad tissue tropism. However, the efficiency of Ad5 gene transfer may closely correlate with the cell surface density of its primary receptor, CAR (4–6). Unfortunately, the expression of CAR is highly variable, and often low, on ovarian and other primary cancer cells, resulting in relative resistance to Ad5 infection (7–11). On the basis of this concept, strategies to modify Ad tropism to circumvent CAR deficiency have used heterologous retargeting complexes or genetic capsid modifications (12). A relatively unexplored variation of the latter approach is the substitution of the knob domain of Ad5 with knobs from alternate Ad serotypes (13–16). Specifically, Ad3 has a distinct, but unidentified receptor and, therefore, different tissue tropism (17, 18).

In this study, we have constructed an Ad containing a chimeric fiber with the knob domain of Ad3 in the Ad5 capsid (Ad5/3luc1), which redirects binding of the vector to the Ad3 receptor. We show that this genetically modified Ad vector significantly enhances the infectivity of human ovarian adenocarcinoma cell lines and primary human ovarian cancer cells. Also, our results suggest correlation between transgene transfer and receptor expression as measured by a novel knob binding assay. Genetic retargeting with the Ad3 knob may allow more efficient tumor cell transduction in the context of *in vivo* gene delivery and, thus, may offer the potential to improve Ad-based ovarian cancer gene therapy approaches.

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[2] To whom requests for reprints should be addressed, at Division of Human Gene Therapy, Gene Therapy Center, The University of Alabama at Birmingham, 1824 Sixth Avenue South, WTI 620, Birmingham, AL 35294-3300. Phone: (205) 934-8629; Fax: (205) 975-7476; E-mail: David.Curiel@ccc.uab.edu.

[3] The abbreviations used are: Ad, adenovirus; CAR, coxsackie and adenovirus receptor; VP, viral particle; DMEM:F12, DMEM:Ham's F-12; FBS, fetal bovine serum.

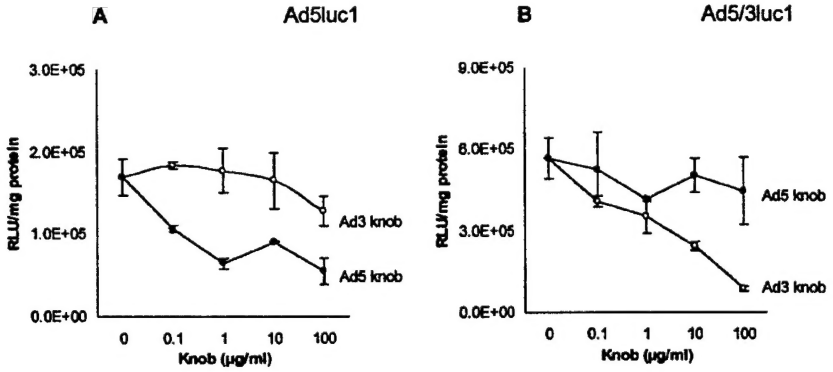

Fig. 1 A chimeric Ad with the knob from serotype 3 binds to the Ad3 receptor. SKOV3.ip1 cells were preincubated with the indicated concentrations of recombinant Ad5 (●) or Ad3 (○) knob. Ad5luc1 (*A*) or Ad5/3luc1 (*B*) was added at 5000 or 200 VP/cell, respectively, and luciferase activity was measured after 24 h. Luciferase activity is expressed as relative light units (*RLU*) normalized for total protein concentration. Each *point* represents the mean of three experiments ± SD.

MATERIALS AND METHODS

Cells and Tissues. Human ovarian adenocarcinoma cell lines OV-4, SKOV3.ip1, and Hey were obtained from Dr. Timothy J. Eberlein (Harvard Medical School, Boston, MA), and Dr. Janet Price and Dr. Judy Wolf (both from M. D. Anderson Cancer Center, Houston, TX), respectively. The human ovarian teratocarcinoma cell line PA-1 was obtained from the American Type Culture Collection (Manassas, VA). The 293 human transformed embryonal kidney cell line was purchased from Microbix (Toronto, Ontario, Canada). All cell lines were cultured at 37°C in media recommended by suppliers in a humidified atmosphere of 5% CO_2.

Fresh malignant ascites fluid samples from patients with pathologically confirmed ovarian adenocarcinoma were obtained from the University of Alabama at Birmingham Hospital. Cancer cells were purified by a previously described immunomagnetic-based method (19). Briefly, ovarian cancer cells were initially bound with a murine anti-TAG-72-antibody (CC-49) and then collected with magnetic beads coated with antimouse-IgG.

Recombinant Ads. Two replication-incompetent Ad vectors containing a firefly luciferase transgene cassette in place of the deleted E1 region were used. Ad5luc1 was generated in our laboratory and described previously (20). The genome of Ad5/3luc1 (Ad containing chimeric fibers with the tail and shaft domains of Ad serotype 5 and the knob domain of serotype 3) was constructed by homologous DNA recombination in *Escherichia coli* using the previously described plasmids pNEB. PK.F5/3 (13) in a scheme described by Dmitriev *et al.* (21). The vector of interest was rescued by transfecting 293 cells with the resultant Ad genome. The viruses were propagated on 293 cells and purified on cesium chloride gradients. The VP concentration was determined at 260 nm, and a standard plaque assay on 293 cells was performed to determine infectious particles. The ratio of VP:infectious particles was 5.24 and 45.7 for Ad5luc1 and Ad5/3luc1, respectively.

Recombinant Fiber Knob Proteins. Recombinant Ad5 and Ad3 fiber knob proteins with N-terminal 6xHis tags were expressed in *E. coli* using the pQE30 expression vector (Qiagen, Valencia, CA) and purified on nickel-nitrilotriacetic acid agarose columns (Qiagen) as recommended by the manufacturer and described elsewhere (13). The concentration of the purified proteins was determined by Bio-Rad DC protein assay (Bio-Rad, Hercules, CA). The ability of each knob protein to form a homotrimer was verified by Western blot of unboiled samples. The primary antibody used in detection was Penta-His antibody (Qiagen) and the secondary antibody was peroxidase-conjugated goat antimouse IgG (Sigma Chemical Co., St. Louis, MO).

Competitive Binding Assay. To investigate the ability of recombinant Ad5 and Ad3 knobs to block infection by the Ad of the corresponding serotype, infection with Ad5luc1 and Ad5/3luc1 was performed in the presence of the purified knob proteins. Monolayers of SKOV3.ip1 cells in 24-well plates were preincubated with increasing concentrations of Ad5 or Ad3 knob in 100 µl of DMEM:F12 with 2% FBS for 10 min at room temperature. Ad5luc1 or Ad5/3luc1 was added at 5000 or 200 VP/cell, respectively, diluted in 100 µl of DMEM:F12 with 2% FBS, followed by a 30-min incubation at room temperature. The cells were then washed once with DMEM:F12 containing 2% FBS, and complete medium was added. After 24 h of incubation at 37°C, the cells were lysed and a luciferase assay was performed with the Luciferase Assay System (Promega, Madison, WI). The protein concentration of the cell lysates was determined as above to allow normalization of the gene expression data for the number of cells. Background luciferase activities were subtracted from the readings.

Determination of Receptor Expression by Flow Cytometry. Cells grown in T75 flasks were washed with PBS, harvested by incubating with 0.53 mM EDTA in PBS, and resuspended in PBS containing 1% BSA. Cells (2×10^5) were incubated with 20 ng of either Ad5 or Ad3 recombinant knob protein in 200 µl of PBS-BSA, or with buffer only, for 1 h at 4°C. Cells were washed twice with 4 ml of PBS-BSA and incubated with 300 µl of a 1:125 dilution of Tetra-His antibody (Qiagen) for 1 h at 4°C. The cells were washed once with 4 ml of PBS-BSA and incubated with 300 µl of a 1:100 dilution of the secondary FITC-labeled goat antimouse IgG (Sigma Chemical Co.) for 1 h at 4°C. After the cells were washed as described above, 2.6 µg/ml propidium iodide (Sigma Chemical Co.) was added to sort out dead cells from the sample; then, 2×10^4 cells (OV-4) or 10^4 cells (other cell lines) were analyzed immediately by flow cytometry at the University of Alabama at Birmingham FACS Core Facility. The FITC-positive (live) cell population for each cell line was determined by gating cells incubated with buffer only (negative control) at 1%.

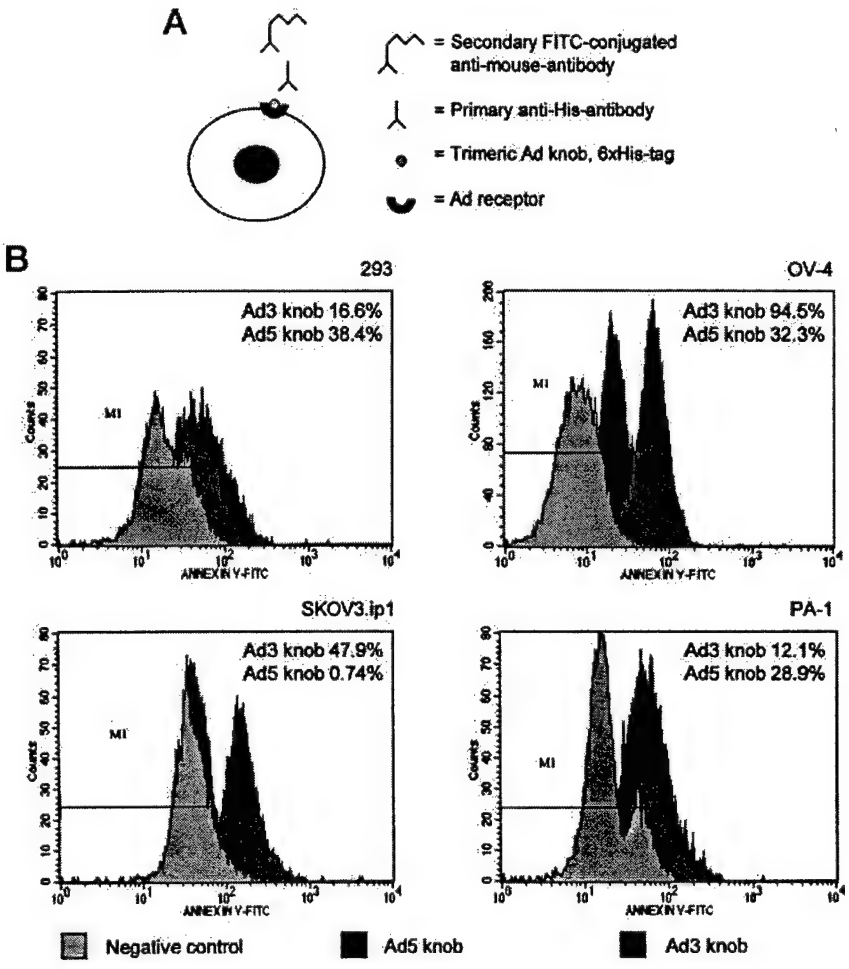

Fig. 2 Differential expression of Ad serotype 5 and 3 receptors on ovarian adenocarcinoma cell lines. Cells were incubated with 10 ng/100 μl of recombinant Ad5 or Ad3 knob protein, then with a primary Tetra-His antibody, and then with a secondary FITC-labeled goat antimouse antibody (*A*). In the negative control (without knob), cells were incubated with primary and secondary antibodies only. Aliquots of 2×10^4 (OV-4) or 10^4 cells (other cell lines) were analyzed by flow cytometry. Shown is the binding of Ad5 knob (*black areas*) and Ad3 knob (*gray areas*) to 293, OV-4, SKOV3.ip1, and PA-1 cells (*B*). The *light gray areas* represent cells incubated without knob protein (negative control).

Ad-mediated Gene Transfer Assays. Cells in 24-well plates were infected for 30 min at room temperature at 50, 200, and 1000 VP/cell by adding Ad5luc1 or Ad5/3luc1 diluted in 200 μl of DMEM:F12 with 2% FBS. Cells were washed once with DMEM:F12 containing 2% FBS, and complete medium was added. The luciferase assay was performed 24 h postinfection as described above.

RESULTS AND DISCUSSION

Due to variable expression of CAR on human primary cancer cells, the utility of Ad5 as a cancer gene therapy vector could be compromised. Fortunately, native Ad5 tropism can be modified to circumvent CAR deficiency and to enhance infectivity. One approach is retargeting Ads by creating chimeric fibers possessing knob domains of alternate serotypes. To this end, we constructed nonreplicating Ads with either the native Ad5 fiber protein (Ad5luc1) or a chimeric fiber with the knob from Ad3 fiber (Ad5/3luc1). To evaluate the receptor binding properties of Ad5/3luc1 in comparison with Ad5luc1, infections of SKOV3.ip1 cells were performed in the presence of purified, trimeric recombinant Ad5 and Ad3 knob proteins (Fig. 1). Transgene expression mediated by Ad5luc1 decreased with increasing concentrations of Ad5 knob in a dose-dependent manner. In contrast, the purified Ad3 knob exhibited only limited ability to block the infection with Ad5luc1 (Fig. 1*A*). Similarly, Ad3 knob blocked Ad5/3luc1 infection, whereas Ad5 knob had only minimal effect (Fig. 1*B*). The data obtained in this competition experiment demonstrate that the relevant recombinant knob protein is able to block the infection of SKOV3.ip1 cells with either Ad5luc1 or Ad5/3luc1 in a dose-dependent manner, whereas gene transfer is only minimally inhibited by high concentrations of the irrelevant knob. Therefore, our results support the existence of a distinct receptor for Ad3, as suggested previously (13, 14, 17, 18). More importantly, these results confirm that Ad5/3luc1 is retargeted to the Ad3 receptor.

We sought to establish that the level of Ad3 receptor would predict sensitivity of cells to the chimeric vector. Because the Ad3 receptor is not yet identified, we developed a novel knob binding assay to quantify the cell surface expression of CAR and the Ad3 receptor on human ovarian cancer cell lines (Fig. 2*A*). For this analysis cells were incubated with recombinant, 6xHis-tagged Ad5 knob or Ad3 knob, followed by detection

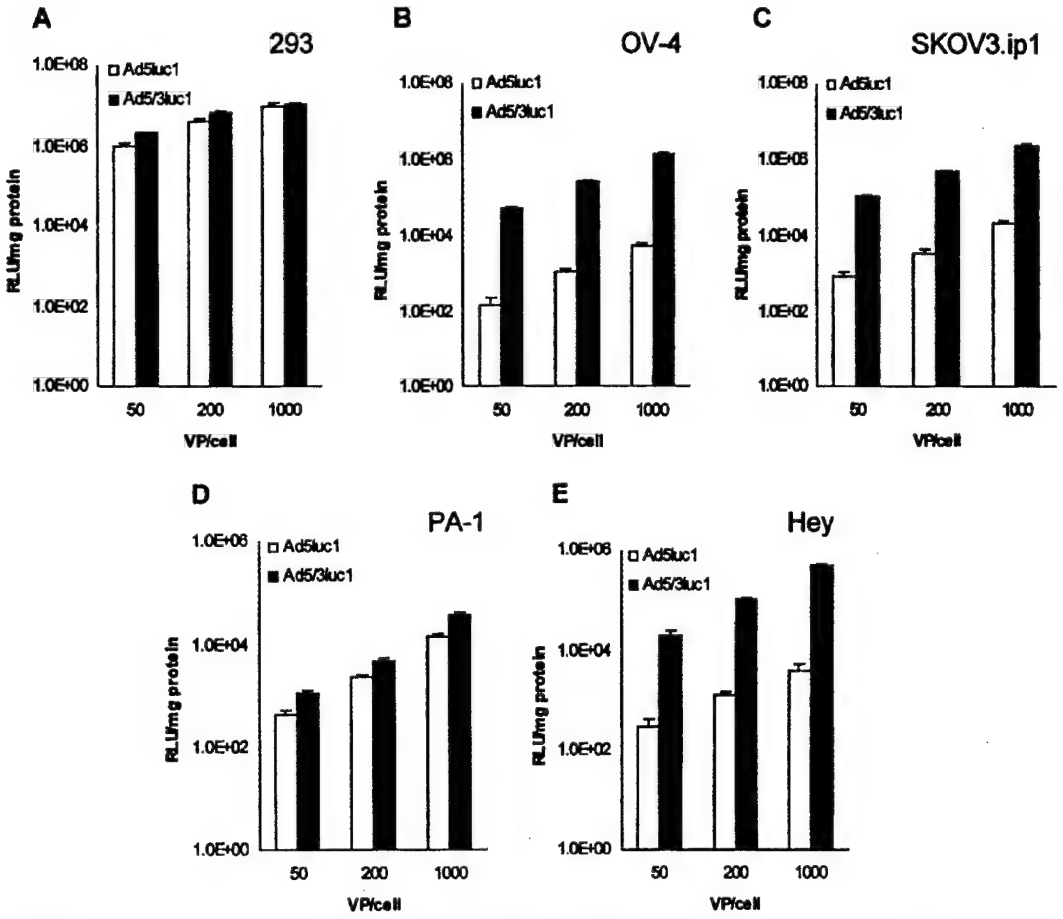

Fig. 3 A chimeric Ad with the knob from serotype 3 displays increased infectivity for human ovarian cancer cell lines. 293 (*A*), OV-4 (*B*), SKOV3.ip1 (*C*), PA-1 (*D*), and Hey (*E*) cells were infected with Ad5luc1 (□) and Ad5/3luc1 (■) at 50, 200, and 1000 VP/cell, and luciferase activity was measured after 24 h. Luciferase activity is expressed as relative light units (*RLU*) normalized for total protein concentration. Each *point* represents the mean of three experiments ± SD.

with anti-His antibody and FITC-conjugated antibody. PA-1 cells have been shown to express high levels of CAR (10). In contrast, OV-4 and SKOV3.ip1 display moderate or low levels of CAR (10, 22). The 293 cells were included as a CAR-positive control (21). This knob binding assay suggested that 293 cells express larger amount of CAR than Ad3 receptor (Fig. 2*B*). Also, PA-1 displayed more CAR than the Ad3 receptor on the cell surface. In contrast, human ovarian adenocarcinoma cell lines OV-4 and SKOV3.ip1 expressed more Ad3 receptor than CAR. These results suggest higher expression of the Ad3 receptor relative to CAR on human ovarian adenocarcinoma lines (PA-1 is a teratocarcinoma).

We hypothesized that differential expression of CAR and Ad3 receptor could correlate with infectivity by serotype 5 or chimeric virus. Infection at 1000 VP/cell of Ad5/3luc1 resulted in 1.12-, 291-, 125-, 2.60-, and 116-fold higher luciferase expression in 293, OV-4, SKOV3.ip1, PA-1, and Hey cells, respectively, in comparison with Ad5luc1 (Fig. 3). The relative differences were very similar to the other amounts of virus. Non-adenocarcinoma cell lines expressing similar levels of CAR and Ad3 receptor in the knob binding assay (293 and PA-1) demonstrated comparable results in the gene transfer assay. In contrast, transgene expression in adenocarcinoma cell lines (OV-4, SKOV3.ip1, and Hey) was 2 orders of magnitude higher with Ad5/3luc1. Importantly, the receptor density, as estimated by the novel knob binding assay, correlated with reporter gene expression. The actual transgene expression in 293 cells was higher, perhaps resulting from E1 trans-complementation and viral replication. Thus, the amount of binding to primary receptor appears to be the major factor determining the efficiency of Ad-based gene delivery to target cells. Nevertheless, there are other receptors that could be sufficient to mediate the initial binding of Ad5 (23). Furthermore, expression of αv integrins may affect the infectivity of cells by Ad.

Human trials have suggested a discrepancy between cell line and clinical gene transfer efficiency (24, 25). To more closely model the clinical situation with the most stringent available substrate, gene transfer experiments were performed using unpassaged human primary ovarian adenocarcinoma cells, purified from malignant ascites fluid. Recent studies have

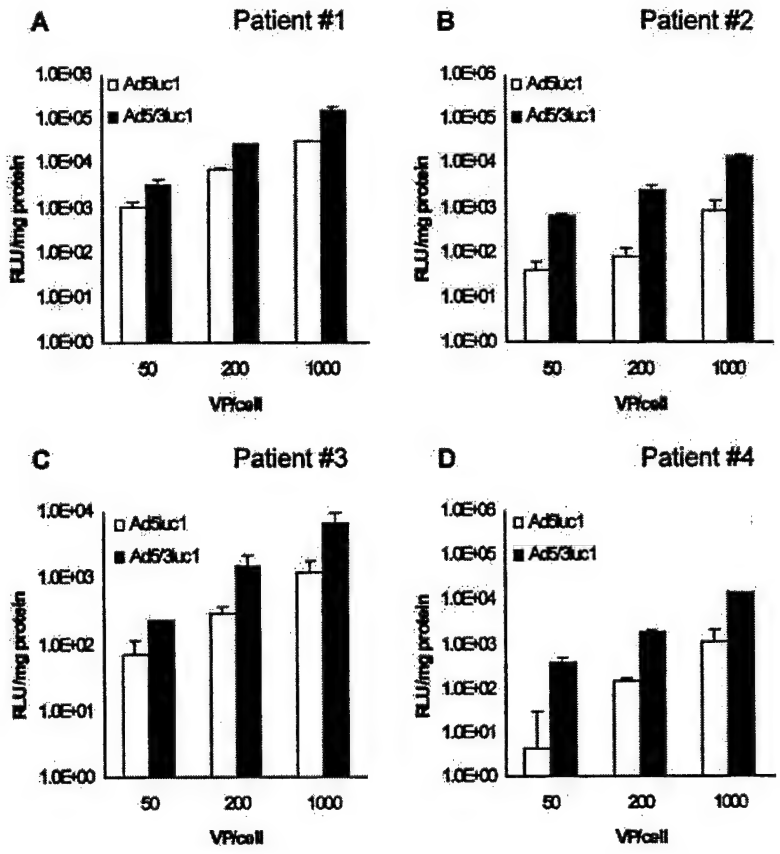

Fig. 4 A chimeric Ad with the knob from serotype 3 displays increased infectivity for purified human primary ovarian cancer cells. Cells were infected with Ad5luc1 (□) and Ad5/3luc1 (■) at 50, 200, and 1000 VP/cell, and luciferase activity was measured after 24 h. Luciferase activity is expressed as relative light units (*RLU*) normalized for total protein concentration. Each *point* represents the mean of three experiments ± SD.

shown that most primary ovarian cancer cells display moderate to low levels of CAR (10). In our experiments, ovarian cancer primary cells obtained from four patients demonstrated 5.18-, 16.5-, 5.63-, and 12.5-fold higher transgene expression when infected at 1000 VP/cell of Ad5/3luc1 in comparison with Ad5luc1 (Fig. 4*A–D*, respectively). Infection at 50 and 200 VP/cell gave similar results. For some samples, the findings were also corroborated at 5000 VP/cell.[4] Thus, an augmentation of luciferase activity was observed with the modified vector, but to a smaller extent than with ovarian adenocarcinoma cell lines. These results underline the necessity of analyzing primary tumor material in addition to established cell lines.

As with ovarian cancer, variable expression of CAR is documented in many other cancer types such as glioma, melanoma, bladder cancer, and rhabdomyosarcoma (7–11, 26). It is known that, for entry, viruses often exploit cellular receptors important in conserved pathways (27). Interestingly, previous studies suggest that CAR may act as a tumor suppressor, which could be linked to the frequent down-regulation seen in highly tumorigenic cells (28). Our results suggest that expression of CAR *versus* the Ad3 receptor is different on human ovarian cancer cells, and the density of Ad3 receptor is often higher. Although the receptor and its function are unknown, it is not impossible that the expression of the Ad3 receptor could be unrelated to the carcinogenic process and therefore unaffected by malignant progression. Thus, Ad3 receptor-mediated gene transfer could be advantageous in the context of advanced cancer.

In conclusion, we describe a chimeric Ad5/3luc1 incorporating the Ad3 knob in the Ad5 fiber. True genetic retargeting, as described here, could give an advantage in comparison with enhanced infectivity, *i.e.*, viruses that continue to bind CAR despite tropism modification. Exploiting the different tropism of Ad3 led to enhanced infectivity of ovarian cancer cell lines and primary cells. Furthermore, we used a novel knob binding assay to investigate receptor concentration and found high expression of the Ad3 receptor on ovarian adenocarcinoma cells.

[4] Data not shown.

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APPENDIX B

Adenoviral Gene Therapy for Renal Cancer Requires Retargeting to Alternative Cellular Receptors

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Renal cell carcinoma (RCC) is resistant to chemotherapy and radiotherapy. Therefore, the identification of new agents with better antitumor activity merits high priority in the treatment of advanced RCC. In this regard, gene therapy based upon adenoviral vectors is a promising new modality for cancer. One limiting factor for the utility of adenoviruses based on serotype 5 for cancer gene therapy is their critical dependence on cellular expression of the primary adenoviral receptor, the coxsackie and adenovirus receptor (CAR). Because CAR deficiency emerges as a universal finding in tumors, we employed genetic approaches to alter the infection tropism of replication-deficient adenoviral vectors to RCC, thereby improving adenoviral infection efficiency. On this basis, we designed replication-competent adenoviral anti-tumor agents which embodied these novel targeting paradigms. These genetic capsid modifications allowed adenoviral infection, replication and cell-killing in RCC *in vitro* and *in vivo*. Based on these studies, we conclude that retargeting of replication-competent Ad viruses to alternative cellular receptors is a promising experimental modality for renal cancer.

Approximately 30,000 new cases of renal cell carcinoma (RCC) are diagnosed each year in the United States and 12,000 die of their metastatic disease annually. Despite extensive evaluation of many different treatment modalities, metastatic RCC remains highly resistant to systemic therapy and the 5-year survival is 5-10%[1-3]. No single agent or combination therapy has consistently shown a response proportion of 20% or higher. While interleukin-2 and interferon-α-based therapies are most commonly used to treat advanced disease, only low response rates are observed, with durable responses of 5% or less. Therefore, the identification of new agents with better antitumor activity merits a high priority in the treatment of advanced RCC. In this regard, gene therapy is a promising new modality for cancer, whereby transfer of immunomodulatory, tumor-suppressor or suicide genes may affect the natural course of tumors. Current gene therapy approaches for RCC are based on harnessing the immune system for therapeutic recognition of RCC tumor-associated antigens[4]. Another emerging gene therapy approach for resistant tumors, involves direct cancer cell killing by replicating viruses. This approach has been previously reported for RCC only in the context of the neurotrophic herpes virus, tested experimentally for all tumors derived from the urinary tract[5]. In this regard, because the efficacy of cancer gene therapy is critically dependent on the infection of target tumors cells[6], the mechanism of vector entry into RCC cells is of primary significance. The cellular tropism of adenovirus (Ad) serotype 5, a principal viral vector for cancer gene therapy, is primarily dictated by CAR recognition[7]. After anchoring at this site by virtue of the knob domain of the fiber capsid protein, the virus achieves internalization via interaction of the capsid penton protein with the integrins αvβ1, αvβ3 and αvβ5, present on target cells.

On this basis, relative deficiency of target cell receptors limits the capacity of Ad vectors to infect the target cell. Therefore, CAR deficiency emerges as a limiting factor for the utility of Ad5 vectors for cancer gene therapy[6,8]. One means to circumvent this biological limitation is the redirection of Ad vectors to target cancer cells via alternative cellular receptors. We and others have previously reported two distinct genetic retargeting approaches to alter the tropism of replication-deficient Ad vectors. First, fiber knob chimera has been endeavored whereby an Ad vector with a serotype 5 fiber and a serotype 3 knob has been derived[9,10]. Second, following the identification of the specific binding of the peptide RGD-4C to various integrins[11], this peptide has been incorporated into the fiber[12], or the knob of Ad serotype 5[13]. Both these approaches for the modification of Ad tropism have proven highly efficient for CAR-independent cellular entry in the context of replication-deficient Ad. In this regard, these approaches are now highly consequential for cancer gene therapy in recognition of the nearly universal finding of CAR deficiency in epithelial neoplasms[8,14,15]. Furthermore, the absence of CAR not only inhibits Ad uptake but is also associated with an invasive cancer phenotype [15,16]. In accordance with these observations, the efficacy of Ad-mediated cancer gene therapy has been limited in preclinical and clinical studies by the resistance of the CAR-deficient tumor cells to Ad infection [17,18].

In addition to their employment for gene delivery purposes, Ad viruses have also been used as replicative agents to achieve direct tumor killing. On this basis, while replication-selective Ad viruses have been proposed to overcome tumor resistance to replication-deficient Ad vectors, CAR-deficiency also represents a major hurdle for viral propagation.

In the latter context, CAR-deficiency would not only limit the efficiency of infection by the initial viral inoculum, but more importantly, the potential therapeutic advantages afforded by viral replication would be negated by poor intratumoral spread of the viral progeny due to failure to infect neighboring tumor cells. In accordance with this concept, Phase I and II clinical trials, in which patients with recurrent squamous cell carcinoma of the head and neck had received direct intratumoral injection of an attenuated replicating Ad virus based on the serotype 5 capsid (ONYX-015), have resulted in clinical benefit in less than 15% of cases. Furthermore, in patients with pancreatic and ovarian tumors, ONYX-015 did not appear to replicate at all[19,20].

Based on these considerations, we have studied CAR expression in RCC cell lines and have found a dramatically low cellular CAR expression. Consequently, we have investigated in this study the hypothesis that the oncolytic potency of replicating Ad virus could be enhanced by a novel approach of genetic retargeting to alternative cellular receptors that we had identified in RCC. These receptors include $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins and the putative receptor to Ad serotype 3. This strategy allowed the first direct evaluation of the utility of tropism-modified, replication-competent Ad viruses as an experimental therapy for RCC. We demonstrate that deficiency of the primary Ad receptor on RCC tumor cells restricts the oncolytic potency of replicating Ad5 viruses, both *in vitro* and *in vivo*. In contrast, tropism modification efficiently improves viral entry into RCC cells, resulting in successful viral replication and cancer cell killing. The superiority of the tropism-modification approaches was dramatically higher in the replication-competent Ad viruses relative to replication-incompetent Ad vectors.

This suggests that the efficacy of currently-employed replicating Ad viruses could be further improved by modifications that allow CAR-independent infection of target cancer cells in general, and RCC in particular.

RCC lines are resistant to infection by Ad serotype 5

Based on their merit as gene delivery vehicles, Ad5 vectors have been used in a variety of cancer gene therapy approaches. However, their utility is restricted in many tumors by the low expression of the primary cellular Ad receptor, CAR[6]. Therefore, we first evaluated in human RCC lines the infection efficiency of unmodified Ad vectors based on the capsid of Ad serotype 5. For these studies, we used Ad5luc1, a replication-deficient Ad encoding a CMV-driven luciferase reporter gene, as a means to quantitatively determine infection efficiency. We compared RCC lines to the lung cancer cell line A549 which has an intermediate infection efficiency by unmodified Ad5 vectors[21]. Ad5luc1 was delivered to the cells in muliplicity of infection (MOI) of 100 or 1000 viral particles (vp) per cell, and 48 h later, cells were evaluated for luciferase gene expression. In these studies, while the control A549 cell line demonstrated high levels of Ad5-mediated gene delivery, the human RCC lines were all relatively resistant to Ad5 infection (Fig. 1*a*). These experiments confirmed the inadequacy of replication-deficient Ad5 for gene delivery into RCC lines. Next, we hypothesized that the resistance of RCC lines to Ad5 vectors is also maintained in the context of replication-competent Ad5. To this end, we infected a variety of RCC lines with replication-competent Ad5 viruses, either containing the E3 region (wild type Ad5 {Adwt}), or E3-deleted (Ad5luc3).

We observed in these experiments that even after prolonged periods, viral cytopathic effect (CPE) could not be demonstrated for any of the replication-competent Ad5 vectors in RCC lines. In contrast, these viruses efficiently killed cells of other lineages, such as the human lung cancer cell line A549, and the human cervical cancer cell line HeLa, both known to express CAR (Fig. 1*b*). Next, we hypothesized that the difference in the cell killing properties of the replication-competent Ad5 virus may be accounted for by different patterns of viral infection and replication in the various cell lines. To study the kinetics of Ad5 replication in RCC and control cell lines, we collected the cell and the media fractions following infection with replicative Ad5 virus and measured Ad5 DNA over time. Evaluation of the viral DNA copy number of the replication-competent Ad5 indicated that Ad5 does not replicate in RCC lines. In contrast, these Ad5 viruses replicated well in CAR-expressing cells (Fig. 1*c*). Because several Ad5 viruses, differing in transcriptional regulation but sharing the Ad serotype 5 capsid, could replicate in CAR-expressing cells but not in RCC lines, the underlying mechanism is compatible with distinct infection patterns stemming from variable expression of Ad receptors. Thus, these studies have demonstrated that while RCC lines are relatively resistant to replication-deficient Ad vectors based on the wild type Ad5 fiber knob, their resistance to replication-competent Ad5 viruses is striking.

Resistance of RCC lines to Ad5 is due to CAR-deficiency

Cellular entry of Ad5 is mediated by initial binding of the Ad5 fiber knob to CAR[7], followed by internalization via interaction of the RGD peptide on the capsid penton base with cellular membrane αv integrins[22]. To establish the biological basis for the resistance of RCC lines to Ad5, we studied the expression of Ad receptors that mediate Ad binding and internalization with indirect flow cytofluorometry, using antibodies specific for CAR, αvβ3 and αvβ5 integrins. Because RCC is derived from the renal proximal tubule, we selected the stably-transformed embryonic renal tubular 293 cell line as a control. We found that while 293 cells expressed high levels of CAR, the renal cancer cells displayed dramatically reduced levels of CAR (Fig 1a, Table 1*a*). We confirmed these findings using knob binding assays as an alternative approach for CAR measurement. In these studies, CAR expression was undetectable in RCC lines, unlike in 293 cells (Fig. 1*b*, Table 1*b*). To determine the cell surface expression of alternative Ad receptors, we measured the expression of αvβ3 and αvβ5 integrins and the putative Ad3 receptor in the same RCC and 293 cell lines. We found in 293 cells that the expression of αvβ3 is extremely low and of αvβ5 is moderately low. In contrast, the cell surface expression of these integrins in RCC lines is upregulated (Fig. 1*a*, Table 1*a*). The expression of the Ad3 receptor was not dramatically altered in RCC lines when compared to 293 cells. However, because the expression of Ad3 receptor was maintained in RCC lines, its relative ratio to CAR was increased (Fig. 2*b,* Table 1*b*). Taken together, these findings provide a biological basis for the resistance of RCC to Ad5 and imply that the cell surface expression of alternative Ad receptors may provide potential targets for CAR-independent Ad infection.

Resistance of RCC to Ad5 can be circumvented by retargeting Ad vectors to alternative cellular receptors

The paucity of the primary Ad receptor CAR on RCC lines mandates the consideration of alternative pathways. To achieve effective Ad infection, we employed genetic approaches to generate two tropism-modified Ad vectors. Redirection strategies to αv integrins and the putative Ad3 receptor were based on genetic modification of the viral fiber knob[9,13]. We employed two types of vectors with retargeting motifs. Specifically, one strategy comprised an RGD4C peptide insertion into the HI loop of the Ad5 fiber knob (Fig. 3*a* and *b*). In the other retargeting strategy, a chimeric Ad5/3 vector was constructed comprising the Ad3 knob and the Ad5 fiber. For each approach, replication-deficient vectors and replication-competent viruses were developed. To evaluate the utility of tropism-modified Ad vectors to infect RCC lines, we compared the infection efficiency of gene delivery by the replication-incompetent, tropism-modified vectors AdRGDluc1 and Ad5/3luc1, relative to the replication-incompetent, non-tropism modified Ad5luc1 (Fig. 3*c, d, e* and *f*). In accordance with the observed CAR deficiency in RCC, gene delivery by the non tropism-modified Ad5luc1, was comparable to control mock-infected cells. In contrast, all RCC lines tested demonstrated the superiority of AdRGDluc1 and Ad5/3luc1. We further confirmed the utility of tropism-modified vectors for RCC with real-time determination of gene expression in live RCC cells (Fig. 3*g, h, i* and *j*). Thus, tropism-modified Ad vectors can achieve CAR-independent cellular entry in RCC lines. Based on these alternate cellular retargeting approaches, dramatic enhancement in the efficiency of gene delivery was achieved.

Replication-competent, tropism-modified Ad viruses efficiently infect and kill RCC lines *in vitro*

To evaluate the utility of tropism-modification for RCC killing, we employed replication-competent Ad viruses. Replication-competent Ad viruses have been suggested as a means to overcome the multidimensional structure of tumors that impairs the utility of replication-incompetent vectors for cancer[23,24]. Consequently, we hypothesized that replication-competent, tropism-modified Ad viruses would further increase the therapeutic effect of tropism-modification in RCC. To this end, we tested RCC killing *in vitro* indirectly with gene expression assays and directly with crystal violet assays. Specifically, RCC lines were infected with replication-competent Ad viruses, either tropism-modified or based on the Ad serotype 5 capsid, and assayed for luciferase expression (Fig. 4*a*). The replication-competent, tropism-modified Ad5/3luc3 resulted in a reduction of 2-3 orders of magnitude in gene expression in RCC lines, when compared to the replication-deficient tropism-modified, replication-deficient Ad5/3luc1. Because we also observed diffuse cytopathic effect in RCC lines infected with Ad5/3luc3, we relate the dramatic reduction in gene expression to the oncolytic effect of Ad5/3luc3. This dramatic impact of the combination of replication potency and tropism-modification was not observed in control cell lines (Fig. 4*a*). To further evaluate the cell killing properties of the replicating tropism-modified Ad viruses, we infected RCC lines with either Ad5/3luc3 or AdwtRGD. These replicating viruses differ in the presence of E3 region (E3 is replaced in Ad5/3luc3 by CMV-driven luciferase). As controls, we infected RCC lines with the matching, non-capsid modified, replication-competent Ad viruses, Ad5luc3 and Adwt, respectively.

In these experiments, we observed that only the tropism-modified viruses could kill the RCC lines, while viral CPE could not be demonstrated following infection with the unmodified replicative Ad5 viruses (Fig. 4*b*). Next, we hypothesized that the selective RCC killing capacity of Ad5/3luc3 and AdwtRGD stems from their selective infection and replication in RCC lines.

To evaluate viral kinetics, we measured Ad DNA in cells and media collected from RCC lines infected with either replication-competent, tropism-modified Ad viruses, or the matching replicative Ad5 viruses as controls. While the tropism-modified viruses replicated efficiently, the non-modified Ad5 viruses did not replicate in RCC lines, indicating cellular resistance to infection (Fig. 4*c* and 4*d*). Electron microscopy confirmed that new virion formation in Ad-infected RCC lines was restricted to the tropism-modified Ad viruses (Fig. 4*e* and 4*f*). Thus, these studies have established that the mechanism of CAR-independent RCC killing, using the strategy of retargeting replication-competent Ad viruses to alternative cellular receptors, involves selective viral infection and replication.

Superiority of retargeted Ad viruses in a human RCC model *in vivo*

To study the potency of retargeted Ad viruses in the context of human RCC tumors *in vivo*, we used two different methods. First, we preinfected the RCC CaKi-1 cells with either the replicative control virus Ad5luc3, or the replicative tropism-modified chimeric virus Ad5/3luc3, and then injected the cells subcutaneously into the flanks of athymic nude mice. While Ad5luc3-treated CaKi-1 cells formed progressive tumors, the Ad5/3luc3 treated-CaKi-1 cells did not form tumors (Fig. 5*a*, *b* and *c*).

To further evaluate the utility of replication-competent, tropism-modified Ad viruses for an established RCC tumor *in vivo*, we used a previously reported model for a human RCC in athymic mice[25]. This model allows a relatively uniform tumor growth following surgical implantation of comparable pieces of pre-established, histologically intact tumors. These tumor fragments include matrix and vascular supply, thereby quickly forming subcutaneous tumors within several days. When reaching a volume of 80 mm^3, the tumors were injected with Ad5luc3 or the chimeric Ad5/3luc3, in three divided doses of $1x10^9$ viral particles. Partition of the viral dose was required to improve the therapeutic outcome relative to a single injection[26]. Following three weekly injections, only the replicative chimera Ad5/3luc3 could significantly limit the growth rate of pre-established CaKi-1 tumors (Fig. 5*d*). With time as a continuous variable, it was shown that the difference in tumor size at baseline between groups injected with Ad5/3luc3 or Ad5luc3 was not significant (P=0.0952). However, the difference over time (slope) in tumor growth between these two groups was highly significant ($P < 0.0001$). There was also a highly significant difference in growth slopes between Ad5/3luc3 and PBS-injected xenografts ($P<$ 0.0001). There was no significant difference in baseline or growth slope when we compared Ad5luc3 to PBS-injected tumors (P=0.3475, P=0.1122, respectively). Of note, even the replicating tropism-modified Ad virus could not completely eradicate pre-established RCC tumors. This finding may be interpreted in view of the previously reported RCC xenograft invasion by host mouse fibroblasts building up solid strands of connective tissue[27,28], that are likely to interfere with intratumoral viral propagation.

To confirm that the Ad5/3luc3-mediated tumor growth inhibition of the RCC xenograft was due to intratumoral spread of the progeny of Ad viruses, tumor sections were analyzed for *de novo* synthesized Ad capsid proteins. Immunohistochemical staining for the newly-synthesized Ad capsid protein hexon, indicated that the chimeric Ad5/3luc3, but not the unmodified Ad5luc3, replicated and disseminated throughout the RCC xenograft (Fig. 5*e* and *f*). Hexon staining was predominantly either nuclear, indicating virion assembly, or perinuclear, indicating localization to the rough endoplasmic reticulum (Fig. 5*g*). Taken together, these studies have shown that the enhanced oncolytic potency of the replicating Ad5/3luc3 was due to intratumoral viral replication and spread.

Discussion:

A major limitation of current cancer gene therapy strategies is the inability of replication-defective Ad vectors to disseminate throughout a solid tumor[26]. Consequently, a novel class of Ad viruses has been proposed to selectively replicate within cancer cells and to release the viral progeny to spread and infect neighboring tumor cells[29]. However, the universal finding of CAR deficiency in primary tumors[8,14,15], may not only limit the initial infection event, but would also restrict the potential therapeutic benefits afforded by viral replication within the tumor cells. Thus, CAR-deficiency may account for the insufficient therapeutic outcome in clinical trials with replication-selective Ad viruses[19].

In this study, we have investigated the hypothesis that Ad retargeting to alternative cellular receptors could circumvent the natural resistance of RCC to infection by Ad5. To address this problem, we employed tropism-modified Ad vectors and viruses that achieved CAR-independent cellular infection in RCC models, following the identification of integrins of the v class and the putative Ad3 receptor as potential receptors. Importantly, in the context of cell killing, the utility of tropism-modification was most prominent for replication-competent Ad viruses, predicated on their capacity to replicate and spread the viral progeny *in vitro* and *in vivo*.

We and others have previously reported genetic retargeting approaches for replication-deficient Ad vectors[9,12,13,30]. A number of studies have reported that replication-incompetent Ad vectors modified by insertion of an RGD peptide into the HI loop, may be superior to non-modified Ad vectors to transduce glioma cells[31,32], ovarian cancer cells[33], and head and neck tumor cells [34].

Additionally, a chimeric replication-deficient Ad vector displaying the Ad3 knob could also overcome the resistance of ovarian cancer cells to infection with an Ad5 vector[10]. In this study, we employed a novel approach of tropism-modification of replication-competent Ad viruses to address experimentally a disease with an extremely poor prognosis. Because metastatic RCC is resistant to all currently available therapeutic agents, novel approaches are warranted. We have shown here that replication-competent, tropism-modified Ad viruses can efficiently infect, replicate and kill RCC tumor cells that are resistant to Ad based on the capsid of serotype 5. However, despite the beneficial therapeutic effect of the replicative tropism-modified Ad viruses *in vivo*, complete eradication of a mouse RCC xenograft could not be achieved. Consequently, it appears that retargeted replicative Ad viruses should be complemented by means to overcome the intratumoral physical barriers limiting viral dissemination throughout the tumor.

A pertinent aspect of this study involves the first direct evaluation of the efficacy of replicating, tropism-modified Ad viruses for renal cancer therapy. A number of studies have reported that primary cancer cells from human patients express only low levels of the primary Ad receptor, CAR, and are therefore poorly infected by Ad 5 vectors. Based on these data and previous studies[6], CAR-dependent restriction of currently available replicating Ad viruses may pave the way for the use of CAR-independent tumor cell infection. Because tumor cells abundantly express αv integrins and the putative receptor for Ad serotype 3, these alternative receptors may be of utility for CAR-independent cancer gene therapy. In the context of renal cancer, αvβ3 which has an important function in tumor angiogenesis[35], is abundantly expressed by RCC in humans[36,37].

As well, the expression of αvβ5 is also selectively upregulated in RCC. In fact, increased expression of αv integrins correlates with the histologic grade of RCC[37-39]. Furthermore, the integrin profile of RCC *in vitro* is maintained *in vivo*[28], thereby underscoring their importance in RCC biology and the relevance of retargeting novel replicative viruses to RCC.

While the concept of tropism-modification of replicative Ad viruses for cancer therapy holds great promise, its direct benefit in the context of CAR-deficiency is yet to be confirmed. In this regard, Shinoura *et al.* have reported that the potency of a replicating Ad virus in glioma cell lines *in vitro* and *in vivo* could be improved 30-fold by the addition of a stretch of 20 lysine residues to the carboxy terminal of the fiber protein, allowing the virus to bind to cellular heparan sulfate receptors[40]. Similarly, Suzuki *et al.* have shown in a CAR-positive cell line that the efficacy of a replicating Ad can be enhanced by incorporating an RGD peptide motif into the fiber protein[41]. However, because in both these studies the unmodified Ad5 viruses achieved significant infection, replication and cancer cell killing, it appears that these models were not completely representative of the CAR-deficiency status of primary tumors. Additionally, when tested *in vitro* in the context of a CAR-negative rhabdomyosarcoma cell line, a replicative tropism-modified Ad virus was relatively inefficient in killing the cancer cells[42], indicating the need to tailor the retargeting strategy to the tumor receptor profile. Taken together, these earlier studies imply that the utility of CAR-independent Ad viral infection and replication, in the context of CAR-negative tumors, merits intensive investigation.

In this study, we have demonstrated extreme resistance of RCC to infection with a variety of Ad vectors and viruses displaying the Ad serotype 5 capsid. Further, we have characterized the distinctive superiority of tropism-modification for Ad vectors, and particularly for replicating Ad viruses, in the context of RCC. This approach may be complemented by other strategies to restrict Ad replication to tumor cells, either by placing the expression of viral genes, most commonly the E1A gene, under the control of tumor- or tissue-specific promoters, or by the complete or partial deletion of viral genes required for replication in normal cells, but not in tumor cells.

In conclusion, we have shown in this RCC model that CAR-deficiency dramatically restricts the utility of Ad5 for RCC. To achieve a significant therapeutic outcome, CAR-independent infection was required, mostly in the context of replication-competent Ad viruses. These findings are highly consequential for the development of cancer gene therapy strategies in general and RCC in particular.

Materials and Methods

Viruses. Generation and characterization of Ad5luc1, Ad5/3luc1 and Ad5lucRGD1 have been described [9,13]. These Ad vectors are replication-deficient, expressing luciferase from the E1 locus and differing only in their cell surface receptor recognition. Ad5GFP and Ad5RGDGFP both express the jellyfish green flourescent protein (GFP), driven by a CMV promoter and differ only in the insertion of an RGD peptide into the HI loop of the Ad5 fiber knob. Ad5luc3 is a replication-competent Ad5 virus, differing from Ad wild-type (Adwt) by the replacement of E3 with the CMV promoter-driven luciferase gene. Ad5RGDwt is an $E1^+$, $E3^+$ Ad serotype 5 with an RGD peptide inserted into the HI loop of the Ad5 fiber knob. Ad5/3luc3 is an $E1^+$, $E3^-$ chimeric Ad, based on Ad5 genome but displaying the Ad serotype 3 fiber knob[9].

Cells, transfections and infections. The human RCC cell lines ACHN, A498, CaKi-1 and SW157 were purchased from the American Type Culture Collection (Rockville, MD) and were grown in RPMI 1640 growth media supplemented by 10% fetal bovine serum, and 2 mM L-glutamine, sodium pyruvate, sodium bicarbonate, glucose and two-fold vitamin solution (Gibco BRL, Grand Island, New York). Cells were grown at 37 °C in a 5% CO2 humidified incubator. SN12C, a highly metastatic RCC line was described before[43], and was grown as above. Infections were performed 24 h after seeding $2x10^5$ cells per well in 12-well plates. For infections, growth medium was replaced by serum-free medium with the index virus at the indicated multiplicity of infection (MOI). An hour later, the infection media was removed, cells were rinsed with PBS, and 5% fetal bovine serum growth media was restored.

The media was not replaced thereafter during the experiment. At the indicated time points, cells and media were collected and analyzed for determination of Ad5 E1 gene copy numbers.

Determination of receptor expression by flow cytometry. RCC cells were rinsed with PBS, harvested by incubating with 0.53 mM EDTA in PBS, and resuspended in PBS containing 1% BSA (Sigma). For antibody incubation, $2x10^5$ cells were incubated with RmcB (1:80), LM609 (1:100) or P1F6 (1:100) for 1 hr at 4^o C. An isotype-matched normal mouse IgG_1 (1:100) was used as a negative control. The cells were then rinsed with PBS-BSA, and incubated with 1:100 dilution of FITC-labeled goat anti-mouse IgG for 1 hr at 4^0C. After another PBS rinse, 2.6 μg/ml Propidium Iodide (Sigma) was added to sort out dead cells from the sample. Next, 10^4 live cells were analyzed immediately by flow cytometry. Relative mean fluorescence intensity (R-MFI) was calculated as the ratio of the mean fluorescence intensity (MFI) of the sample of the interest to the MFI of the corresponding negative control for each cell line. Cell surface expression of CAR and the Ad3 receptor was measured by knob binding assay as described before[10]. Briefly, cells were incubated with 20ng of 6xHis-tagged recombinant Ad5 or Ad3 knob protein, or with buffer only, followed by incubation with 1:125 dilution of Tetra-His-Antibody (Qiagen, Santa Clarita, California), and then with FITC-labeled goat anti-mouse IgG (Sigma Chemical Co.). All the incubations were performed for 1hr at 4 °C. In the negative control, cells were incubated with primary and secondary antibodies without the knob protein. The FITC-positive (live) cell population for each cell line was determined by gating cells incubated with buffer only (negative control) at 1%.

Antibodies. The anti-CAR mAb RmcB was produced using hybridoma purchased from ATCC (Manassas, Virginia). Murine mAb LM609 to αvβ3-integrin and P1F6 to αvβ5-integrin were both purchased from Chemicon (Temecula, California). Normal mouse IgG_1 and fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG were from Sigma Chemical Co.

Recombinant fiber knob proteins. Recombinant Ad5 and Ad3 fiber knob proteins were expressed in *E. coli* using the pQE30 expression vector (Qiagen, Valencia, California), and purified on Ni-NTA agarose columns (Qiagen) as recommended by the manufacturer and described elsewhere[9]. The concentration of the purified proteins was determined by Bio-Rad DC protein assay (Bio-Rad, Hercules, California). The ability of each knob protein to form a homotrimer was verified by Western blot of unboiled samples.

Ad gene transfer assays. To assess the efficiency of reporter gene transfer by Ad vectors into RCC or control cell lines, $1X10^5$ cells were plated in 24-well plates and allowed to adhere overnight at 37 °C. On the next day, media was removed and cell monolayers were washed once with PBS. Cells were infected for one hour at an MOI of either 10, 100 or 1000 viral particles (VP)/cell, with either no virus, Ad5luc1, or the tropism-modified Ad vectors AdRGDluc1, or Ad5/3luc1. Control infections and dilutions were performed in Opti-MEM (Gibco BRL, Grand Island, New York). After one hour, cells were washed once with PBS and overlaid with growth medium. After 48-hr at 37 °C in a 5% CO2 humidified incubator, cells were rinsed with PBS and lysed with 25mM Tris-phosphate (pH 7.8), 2mM dithiothreitol (DTT), 2 mM 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid, 10% glycerol and 1% Triton X-100.

Cell lysates were assayed for luciferase expression in a Berthold luminometer (Bad Wildbad, Germany), using Luciferase assay system (Promega, Madison, Wisconsin) and total protein concentration was determined using the DC Protein Assay (BioRad, Hercules, California) according to the manufacturer instructions. Light emission was detected in live CaKi-1 cells, 48 h after infection with either Ad5/3luc1 or Ad5luc1, as reported previously[44]. GFP expression was detected with a Leica fluorescence stereo microscope equipped with a 50-W mercury lamp, for high-magnification imaging of GFP-expressing RCC cells. Selective excitation of GFP was produced through a D425/60 bandpass filter. Emitted flourescence was collected through a long pass filter on a Hamamtsu cooled-color CCD camera. Images were processed accordingly for contrast and brightness with the Adobe photoshop 6.0 software.

Ad cell-killing assays. $1x10^5$ cells of ACHN, A498, CaKi 1, SW157 or SN12 were plated in 12-well plates. Twenty four h later, growth medium was replaced by serum-free medium with the index virus at the indicated MOI. An hour later the infection media was removed, cells were rinsed with PBS, and 5% fetal bovine serum growth media was restored. The media was not replaced thereafter during the experiment. Once an advanced cytopathic effect (CPE) was observed for any of the wells, simultaneous crystal violet stains were performed.

TaqMan PCR assay. E1a copy number was determined for each sample obtained from collected cells and media as of the first day after infection. Genomic DNA was isolated and cleaned using a Qiagen Tissue Kit (Qiagen, Santa Clarita, California) following instructions of the manufacturer. Concentration of isolated DNA was determined by spectophotometry.

TaqMan primers and probe were design as follows: the forward primer, reverse primer, and 6-FAM labeled probe to amplify the E1a and E4 genes, were designed by the Primer Express 1.0 software (Perkin Elmer, Foster City, CA) following the recommendations of the manufacturer. The sequences of the forward and the reverse E1a primers were AACCAGTTGCCGTGAGAGTTG (anneals between 966 and 986) and CTCGTTAAGCAAGTCCTCGATACAT (anneals between residues 1033 and 1009), respectively, while the TaqMan probe was CACAGCCTGGCGACGCCA (anneals between residues 988 and 1006). With optimized concentration of primers and probe, the components of Real-Time PCR mixture were designed to result in a master mix with a final volume of 10 µl per reaction containing 1X Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 100 nM forward primer, 100 nM reverse primer, 1nM probe and 0.025% BSA. For the assay, 1µl of extracted DNA sample was added to 10 µl of PCR mixture in each reaction capillary. A no-template control received 10 µl of reaction mixture with 1 µl of water. All capillaries were then sealed and centrifuged using LC Carousel Centrifuge (Roche Molecular Biochemicals, Indianapolis, Indiana) to facilitate mixing. All PCR reactions were carried out using a LightCycler^{TM}System (Roche Molecular Biochemicals, Indianapolis, Indiana). The thermal cycling conditions were 10 minutes at 95 °C and 40 cycles of 15 seconds at 95 °C and 1 minute at 60 °C.

Animal models. To determine the effect of viral preinfection in RCC tumor growth, we infected CaKi-1 cells at a confluence of 60-70% with Ad5luc3 or Ad5/3luc3 at an MOI of 1 for 1 hour. Cells were allowed to grow for 24 h and then $5x10^6$ CAKi-1 cells were injected subcutaneously into the flanks of athymic nude mice. To determine the effect of viral injection into pre-established RCC tumors we followed the surgical implantation model for RCC[25]. Briefly, 6-8 week-old female athymic nude/nude mice were injected subcutaneously with $5x10^6$ CaKi-1 cells. The purpose of growing subcutaneous tumors was to generate a stock of histologically intact tumors for the surgical implantation model. When tumors were growing in the log phase, the animals were sacrificed and tumors harvested. The periphery of the tumor tissue was collected following removal of necrotic tissue near the center of the tumors. The tumor tissue was cut into small pieces of one cubic millimeter each, and implanted into athymic mice subcutaneously, under anesthesia with xylazine/ketamine. This method allowed a relatively uniform growth of the RCC tumors. Tumor volume was calculated using the simplified formula for a rotational ellipsoid[45] (0.5 x length x width^2). On reaching 80 mm^3, tumors were injected with 3 weekly 50 µl doses of either PBS (4 mice), Ad5luc3 in PBS (5 mice) or Ad5/3luc in PBS (7 mice). Bidimensional tumor measurements were taken twice a week with calipers. Animals were sacrificed when tumor burden became excessive. Experiments were performed in accordance with federal guidelines for animal care and approved by the Institutional Animal Care and Use Committee.

Immunohistochemistry. RCC xenografts were embedded in OCT compound, frozen, cut into 5 μ-thick sections, and fixed in 4% paraformaldehyde. Tissues were blocked in 1% BSA in PBS for 30 minutes before each antibody incubation. Primary and secondary antibody incubations were for 30 minutes at 37 ^{0}C. Ad5luc3 and Ad5/3luc3 capsid hexon protein in infected CAKi-1 tumor sections was determined by immunohistochemical analysis using polyclonal goat anti-hexon (Chemicon, Temecula, California) as the primary antibody, and a donkey antigoat (Molecular Probes, Eugene, Orgeon) as a secondary antibody. Nuclei were stained with Hoechst 33258 (Molecular Probes, Eugene, Oregon). Images were acquired on a Leitz orthoplan microscope (Leica Inc., Wetzlar, Germany) and processed accordingly for contrast and brightness with the Adobe photoshop 6.0 software.

Statistical Methods. Tumor volume (mm^3) for the three injected RCC xenograft groups, was recorded for 6 weeks. Descriptive statistics (mean, standard deviation (SD), and standard error of the mean (SEM)) on tumor volume were calculated for each measurement in each group. Using two approaches, tests of repeated measures were performed to compare the mean tumor volumes between the following groups: a) Ad5/3luc3 and the control group, Ad5luc3; b) Ad5/3luc3 and PBS; and c) PBS and Ad5luc3. In the first approach, time was assigned as a continuous variable. The second approach set time as a discrete variable. The model with time as continuous provided a parsimonious mathematical form to characterize the evolution of response measure over time. The model with time discrete variables showed whether mean tumor volume for one group was larger or smaller than a second group at each time point.

$P < 0.05$ was considered statistically significant in all of the analyses. All tests were performed using SAS software (version 8.0; SAS Institute; Inc. Cary, North Carolina).

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Table 1

a Cell surface expression of αvβ3 and αvβ5 integrins and CAR using indirect immunoflourescence.

	MFI	%	R-MFI	TFS	Peak
293					
Neg	4.89	1.00	1.00	0.28	3.49
Ad5	24.2	52.0	4.94	20.6	20.2
Ad3	14.0	23.1	2.86	7.8	10.8
CAKI-1					
Neg	5.89	1.00	1.00	0.48	3.68
Ad5	5.88	0.71	1.00	0.34	3.75
Ad3	8.32	2.08	1.41	1.01	6.38
ACHN					
Neg	6.51	1.00	1.00	0.49	3.46
Ad5	6.96	1.90	1.07	0.96	3.89
Ad3	12.7	8.16	1.95	4.20	11.2

b Cell surface expression of CAR and the Ad3 receptor using knob binding assays.

	MFI	%	R-MFI	TFS	Peak
291					
Neg	5.16	1.00	1.00	0.42	4.49
Avb3	6.32	1.44	1.22	0.53	7.91
Avb5	10.9	7.26	2.11	2.78	10.7
CAR	159	97.5	30.9	166	178
CAKI-1					
Neg	5.26	1.00	1.00	0.41	5.47
Avb3	27.5	51.8	5.24	27.4	33.4
Avb5	23.7	44.8	4.50	18.1	27.1
CAR	6.96	2.55	1.32	1.02	8.43
ACHN					
Neg	3.82	1.00	1.00	0.27	2.92
Avb3	25.8	62.1	6.74	29.4	24.1
Avb5	26.9	73.1	7.03	27.9	23.3
CAR	8.35	10.3	2.19	2.89	9.39

Abbreviations: MFI - Mean fluorescence intensity of total cell population. **%** = Percentage of gated positive, FITC positive living cell population for each cell line following gating negative control cells at 1%. **R-MFI** - Relative MFI as a ratio of the MFI of samples of interest to corresponding negative control sample for each cell line.**TFS**-Total flourescence signal. **Peak**-flourescence value of the peak cell population.

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Figure legends

Table 1. Cell surface expression of CAR, the integrins αvβ3 and αvβ5 , and the Ad3 receptor. ***a,*** The human RCC cell lines CaKi-1 and ACHN, or the control 293 cell line were analyzed for receptor density with indirect flow cytometry. Cells were incubated with the monoclonal Antibodies RmcB (CAR), LM609 (αvβ3) or P1F6 (αvβ5), followed by detection with FITC-labeled secondary antibody. Results are expressed as the R-MFI, which is a ratio of the sample mean flourescence intensity (MFI) to the MFI of the negative control. ***b***, The same cell lines were analyzed with modified, three step flow cytometry, whereby cells were first incubated with recombinant Ad5 or Ad3 knob, and then with primary and secondary antibodies.

Figure 1. Human RCC lines are resistant to infection with Ad serotype 5. ***a,*** Analysis of infection efficiency of replication-deficient Ad5 in RCC lines. The human RCC lines CaKi-1, ACHN and A498 were infected in triplicates at MOIs of 100 (□) or 1000 (■) with the replication-deficient Ad vector Ad5luc1, expressing luciferase from the E1 locus. The human lung adenocarcinoma cell line A549 served as a control cell line to evaluate the infection efficiency. ***b,*** Analysis of the cell killing potency of replication-competent serotype 5 Ad viruses in RCC cells. CAKi-1 and A549 cells were infected at an MOI of 1 with Ad5luc3 (E1 intact, E3 replaced by luciferase) or Adwt (wild-type Ad). Cells were stained with crystal violet 10 days after infection. ***c,*** Analysis of the replication kinetics of Ad viruses in RCC lines or the control A549 cell line. Cells were infected with the replication-competent Ad5luc3 or Adwt, at an MOI of 1.

Cells and media were collected in triplicates from the different cohorts and subject to quantitative polymerase chain reaction (PCR) analysis of Ad E1a copy number as an index of Ad replication. The A549 cell line was infected with Ad5luc3, (■) while CaKi-1 was infected with either Ad5luc3 (♦) or Adwt (▲). *, $P<0.05$.

Figure 2. Human RCC lines do not express CAR but express alternative Ad receptors. ***a,*** Expression of CAR (red) and the integrins αvβ3 (black) and αvβ5 (blue) was measured in the human RCC lines CaKi-1 and ACHN, or in 293 control cell line with indirect flow cytometry. Cells were incubated with the monoclonal Antibodies RmcB (CAR), LM609 (αvβ3) or P1F6 (αvβ5), followed by detection with FITC-labeled secondary antibody. ***b***, The same cell lines were analyzed with modified, three step flow cytometry, whereby cells were first incubated with recombinant Ad5 knob (red) or Ad3 knob (green), and then with primary and secondary antibodies. Normal mouse serum was used as a control in both experiments (gray line).

Figure 3. Tropism modification of replication-deficient Ad vectors achieves CAR-independent RCC infection. ***a-b,*** Structure of Ad fiber protein and its knob domain and the strategy to alter viral tropism. ***a,*** the fiber protein is a homotrimeric molecule, which consists of three distinct structural domains: the *tail,* the *shaft,* and the *knob*. The knob domain fulfils double duties by maintaining trimerization of the fiber and binding to CAR. ***b,*** Three-dimensional model of the fiber knob domain. The flexible HI loop (*circle*), which connects strands H and I, is exposed outside the knob and, therefore, provides a convenient locale for incorporation of the RGD targeting ligand. ***c-f,*** Analysis of RCC lines infection by Ad vectors expressing luciferase.

RCC monolayers were infected with 10 MOI (blank columns) or 100 MOI (black columns) of the replication-deficient vectors, either unmodified vector Ad5luc1, or the tropism-modified vectors Ad5/3luc1 or AdRGDluc1. Ctrl represents mock-infected cells. The relative light units (RLU) of luciferase/milligram of total cellular protein are shown graphically as the mean of multiple assays. ***g-j,*** Real-time detection of gene expression in live RCC cells infected with replication-deficient Ad vectors. ***g-h,*** CaKi-1 monolayers were infected at an MOI of 10 with the luciferase-expressing vectors, either the unmodified Ad5luc1 (*g*) or the tropism-modified Ad5/3luc1 (*h*). Twenty-four hours later, luciferin was added to the medium, followed immediately with light-detection by a cooled-charged-coupled device (CCCD) camera, connected to a confocal microscope. The light signals were merged with photomicrographs of cell membranes, captured with bright field microscopy. Light signals were pseudocolored into yellow and cell membranes were pseudocolored to blue. All light sources were exclusively detected in cells. ***i-j,*** Real-time detection of flouresence from intact RCC cells infected with replication-deficient Ad vectors. CaKi-1 monolayers were infected at an MOI of 10 with the GFP-expressing vectors, either the unmodified Ad5GFP (*i*) or the tropism-modified AdGFPRGD (*j*). Twenty-four hours later, cells were captured for selective excitation of GFP.

Figure 4. Replication-competent, tropism-modified adenoviruses circumvent RCC resistance to Ad5-mediated cell killing. ***a.*** Analysis of luciferase expression as an indirect index of cell killing by replicative Ad virus. Monolayers of the RCC lines CAKi-1, ACHN and A498 were infected with 10 MOI of either the replication-competent Ad5luc3 (black columns) or the replication-competent, tropism-modified Ad5/3 luc3 (white columns). Ctrl represents mock-infected cells. Forty eight h later, cells were analyzed for luciferase expression. The relative light units (RLU) of luciferase/milligram of total cellular protein are shown graphically as the mean of multiple assays. ***b.*** Direct analysis of RCC cell killing by replication-competent Ad viruses. Monolayers of the RCC line CAKi-1 were infected in two different sets of experiments. The tropism-modified Ad5/3luc3 ($E1^+$, $E3^-$) was tested vs. its control Ad5luc3 ($E1^+$,$E3^-$), and the tropism-modified AdRGDwt ($E1^+$,$E3^+$) was tested vs. its control Adwt ($E1^+$,$E3^+$). Mock represents mock-infected cells. Crystal violet staining was performed once advanced CPE was observed for any of the wells. ***c-d,*** Analysis of the viral replication kinetics as a function of tropism-modification. ***c***, Monolayers of the RCC line CAKi-1 were infected at an MOI of 1 with either the replicative, tropism-modified Ad5/3luc3 (♦), or the replicative, non tropism-modified Ad5luc3 (■). Ad DNA was measured following collection of the cellular and media fractions. The E1a gene copy numbers are shown graphically as the mean of multiple assays. ***d,*** Monolayers of the RCC line CAKi-1 were infected at an MOI of 0.1 with either the replicative, tropism-modified AdwtRGD (●) or the replicative, non-tropism modified Adwt (▲).

e-f, Transmission electron microscopy showing nuclei devoid of viral particles in CAKi-1 cells infected with the replicative, unmodified Ad5luc3, ***e,*** or nuclei containing newly synthesized viral particles in CAKi-1 cells infected with the replicative, tropism-modified Ad5/3luc3, ***f.*** Magnification x7,000. Insert in ***f,*** Assembly of new Ad5/3luc3 virions in CAKi-1 cells, x 20,000. *, $P<0.05$.

Figure 5. *In vivo* superiority of replicative tropism-modified adenovirus in RCC tumor. ***a-c,*** Athymic nu/nu mice were injected with CAKi-1 cells, preinfeced with the unmodified replicative Ad5luc3 *a*, or the tropism-modified Ad5/3luc3, *b*, and monitored for tumor growth, (*c*). ***d,*** Intratumoral injection of Ad5luc3 or Ad5/3luc3 into pre-established RCC tumors. Caki-1 cells were first injected subcutaneously into mice to form tumor stocks. Then, identical tumor pieces were implanted subcutaneously and allowed to grow relatively uniformly. When tumors achieved a volume of 80 mm^3 they were injected with either PBS, (n=4), the replicative unmodified Ad5luc3 (n=5), or the replicative tropism-modified Ad5luc3 (n=7). Tumors were injected in three divided doses at the indicated times (arrows). Means $^{\pm}$SEM of tumor size are shown. ***e-f,*** Immunohistochemical staining of the Ad capsid protein hexon in sections from RCC xenografts infected with Ad5luc3 (***e***), or Ad5/3luc3 (***f***). Foci of hexon-positive cells were identified only in Ad5/3luc3-infected tumors. ***g,*** Double staining for hexon and nuclei in Ad5/3-infected CAKi-1 xenografts with disorganized cellular growth. Hexon stain was predominantly nuclear (indicating virion assembly) or perinuclear (localizing to the rough endoplasmic reticulum). *, $P<0.05$.

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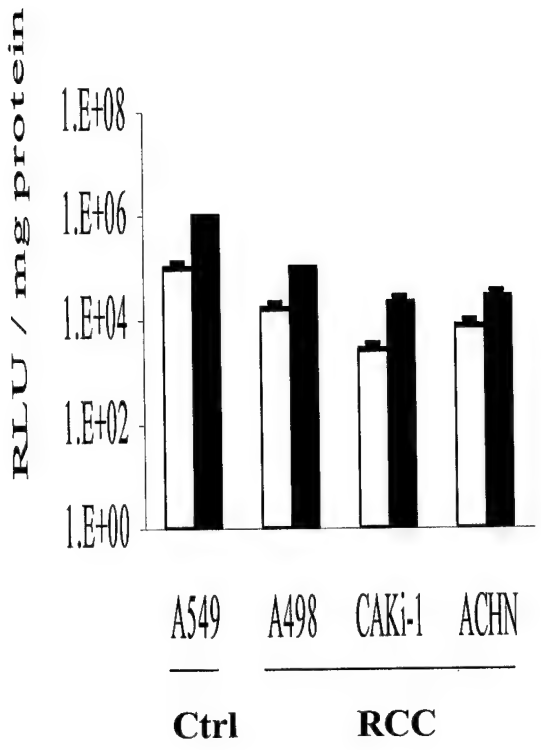
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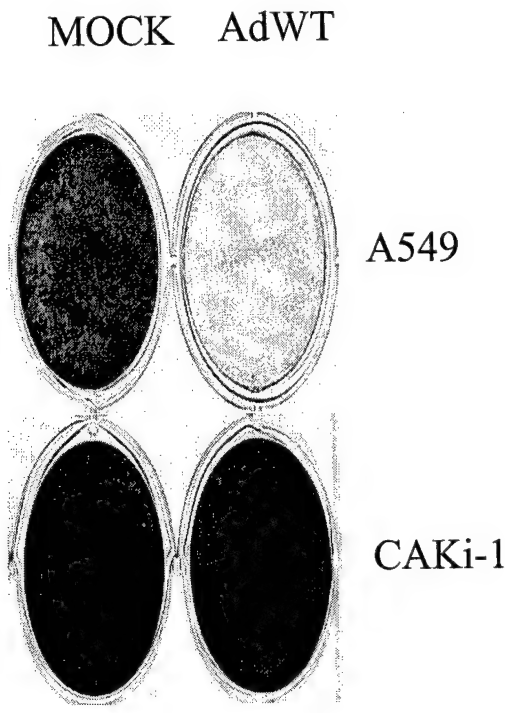
ACHN
Ctrl
RCC

B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AdWT
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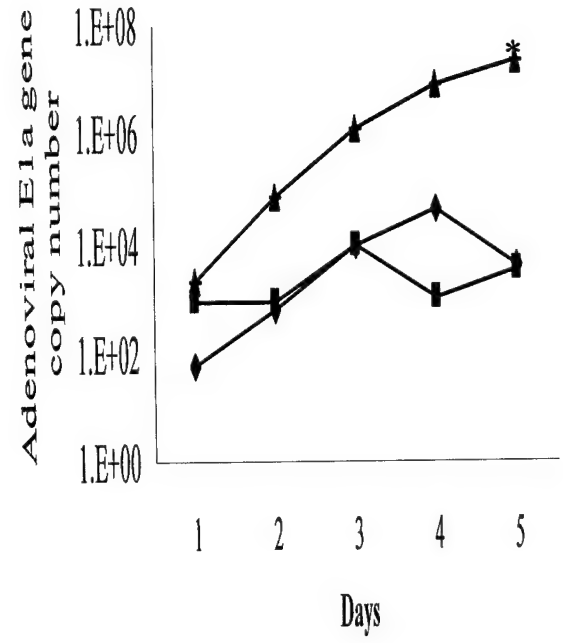
CAKi-1

C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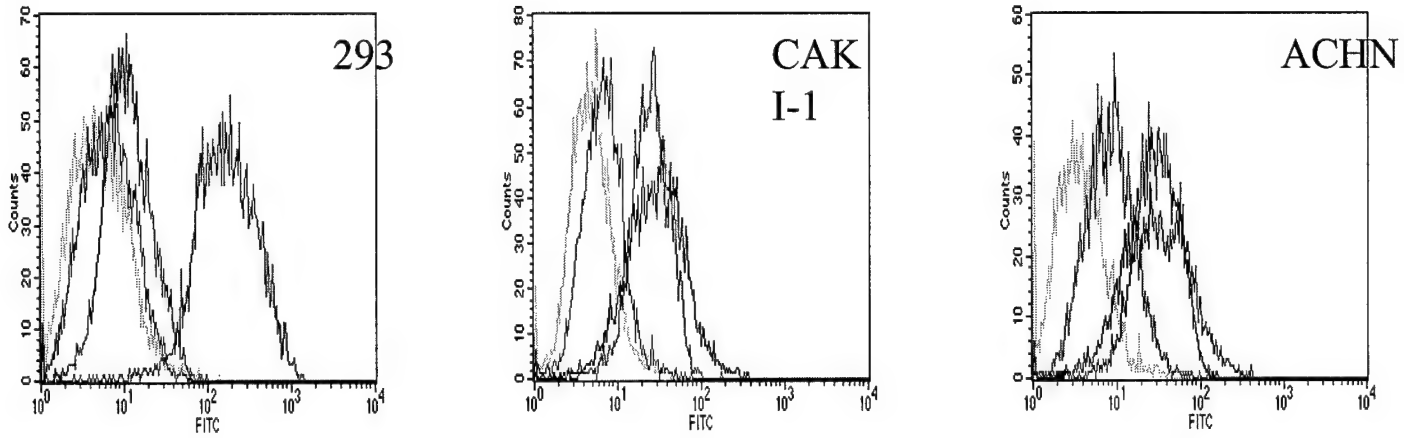
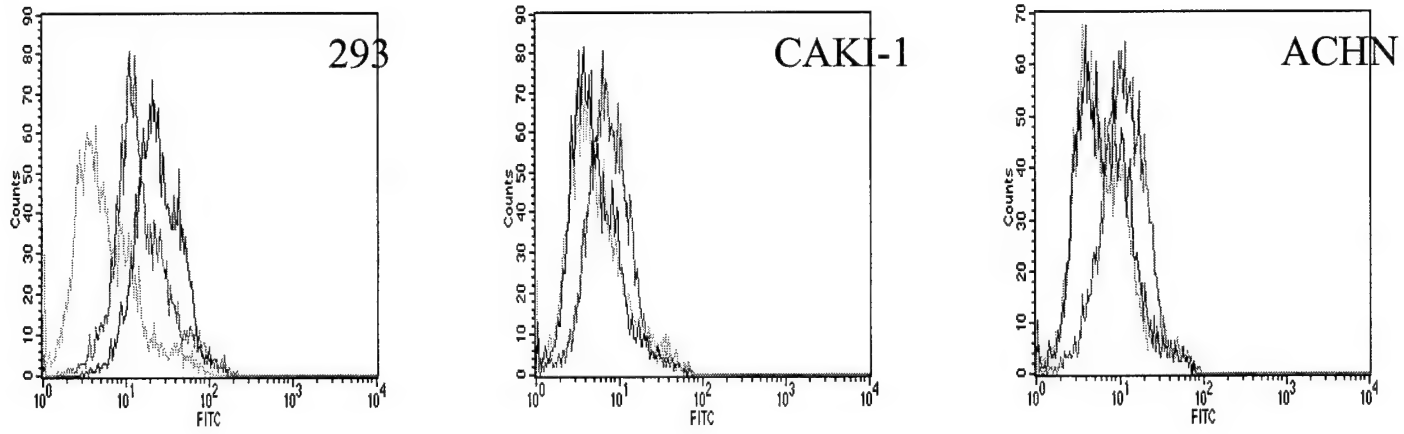
3
4
5
Days

A



Negative control CAR $\alpha_v\beta_3$-integrin $\alpha_v\beta_5$-integrin

B



Negative control Ad5 knob Ad3 knob

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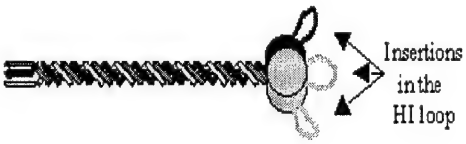
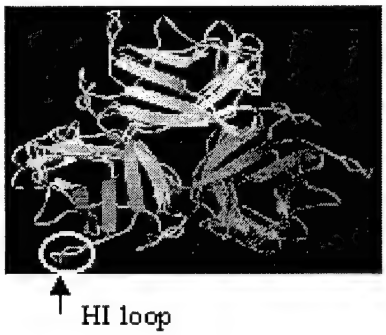

Insertions
in the
HI loop

B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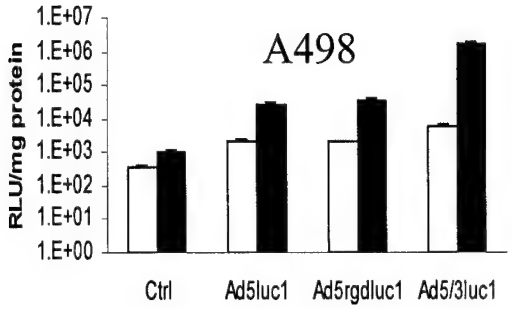
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1.E+00
Ctrl
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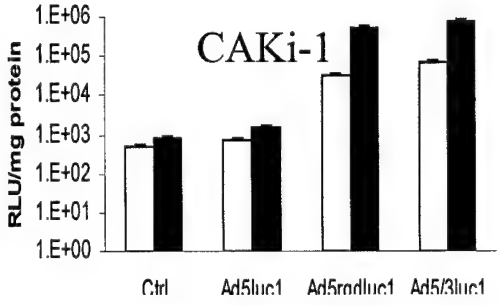
Ad5rgdluc1
Ad5/3luc1

D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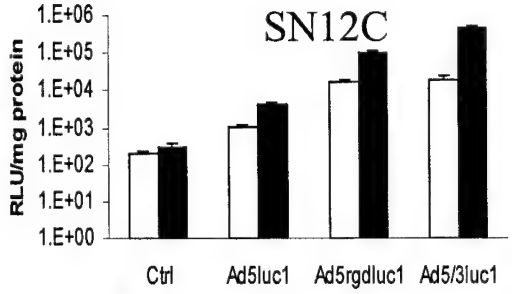
Ad5rgdluc1
Ad5/3luc1

E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Ad5/3luc1

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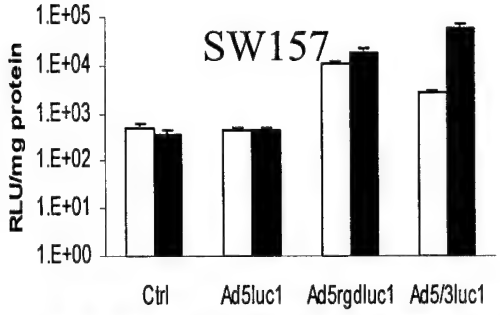
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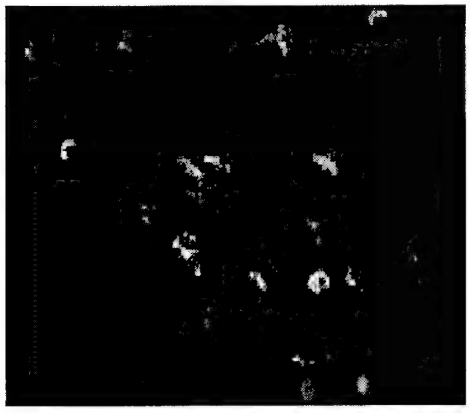
Ad5rgdluc1
Ad5/3luc1

G

H



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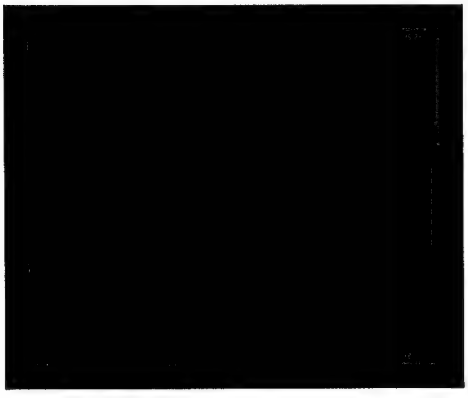

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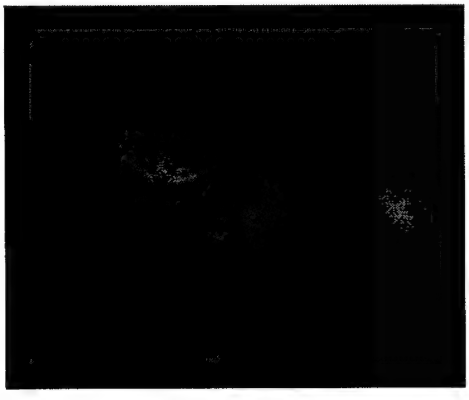

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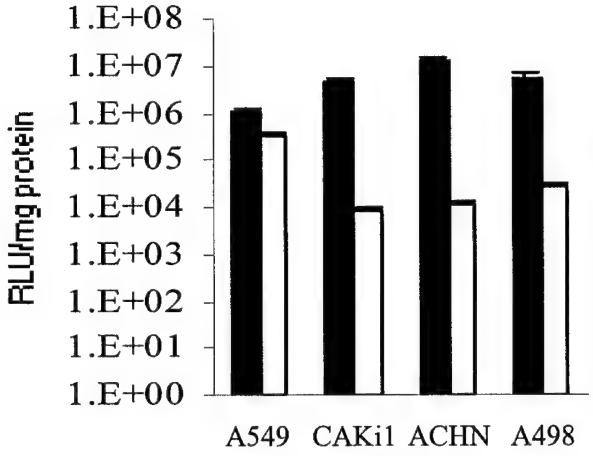
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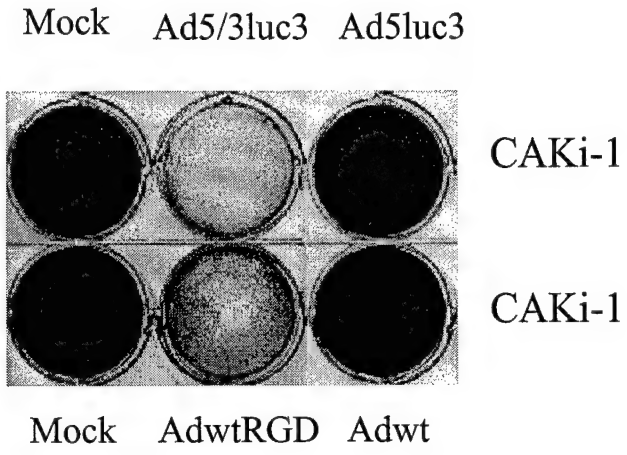
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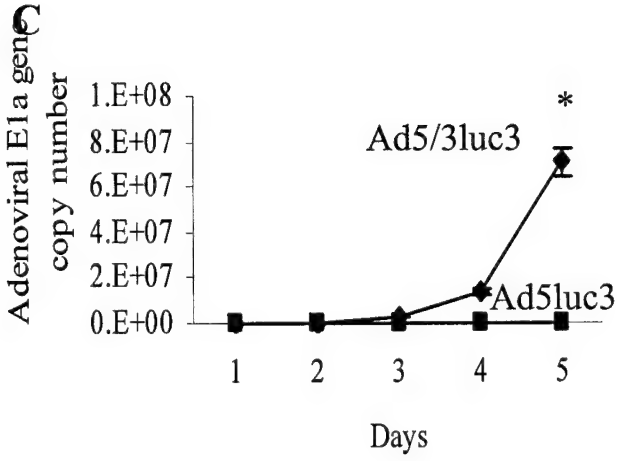
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Ad5luc3
*
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3
4
5
Days

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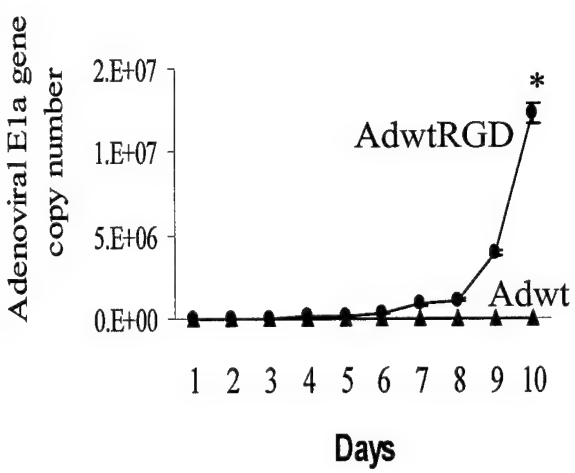
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Adwt
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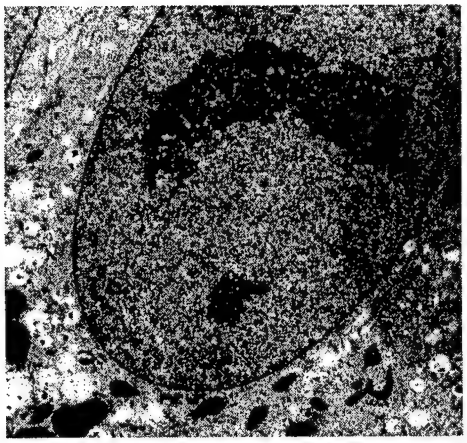
7
8
9
10
Days

E



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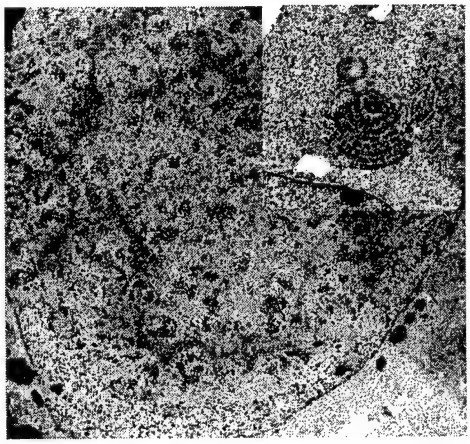

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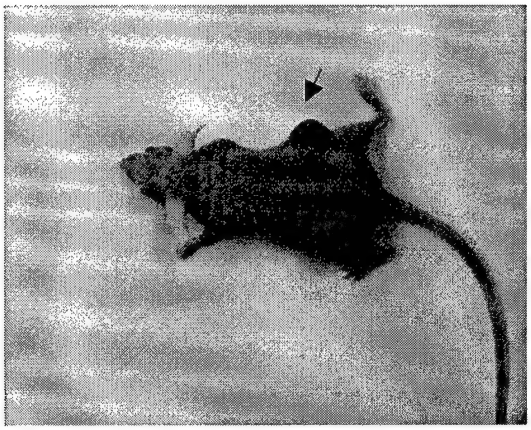

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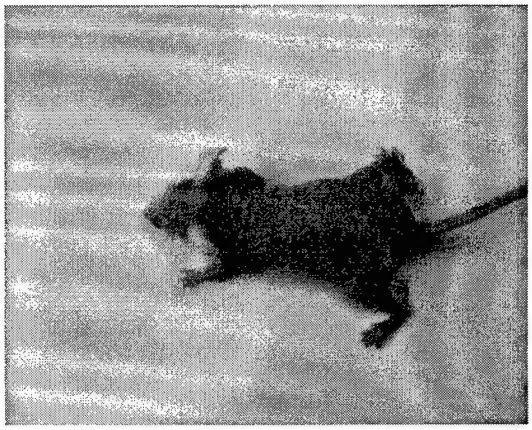

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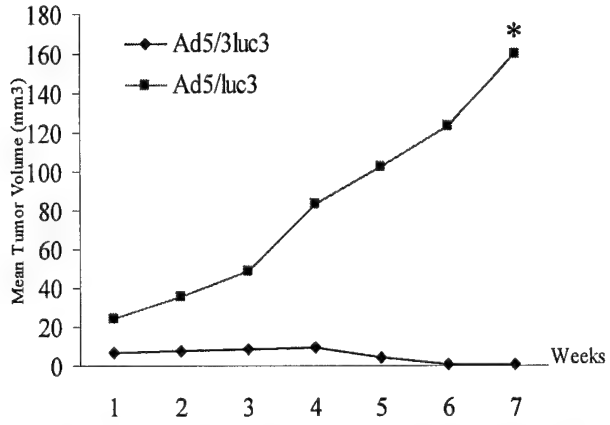
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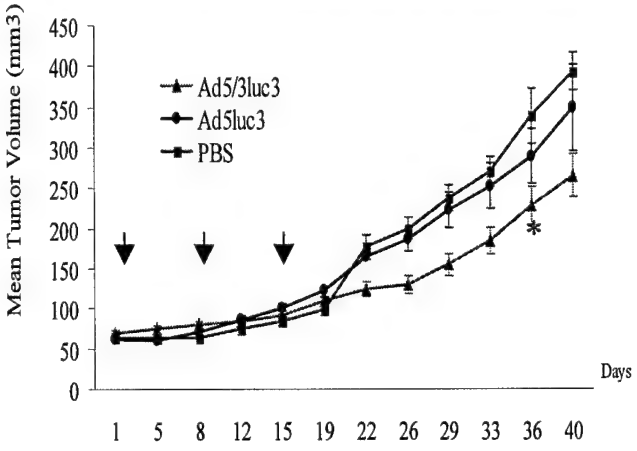
140
120
100
80
60
40
20
0
Ad5/3luc3
Ad5/luc3
*
Weeks
1
2
3
4
5
6
7

D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300
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100
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Ad5/3luc3
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*
Days
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8
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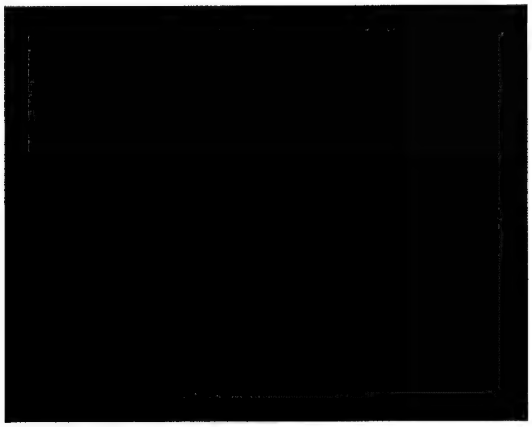
E

F



G

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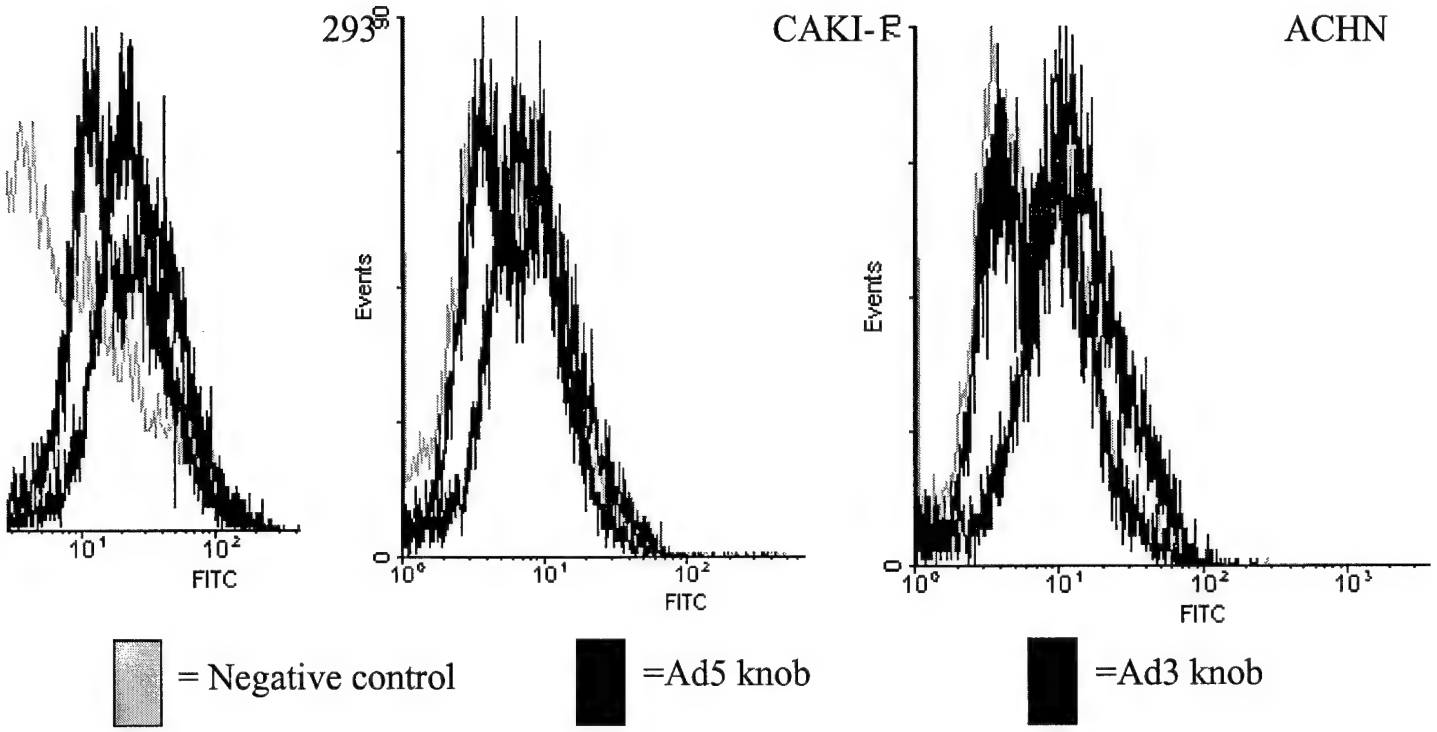
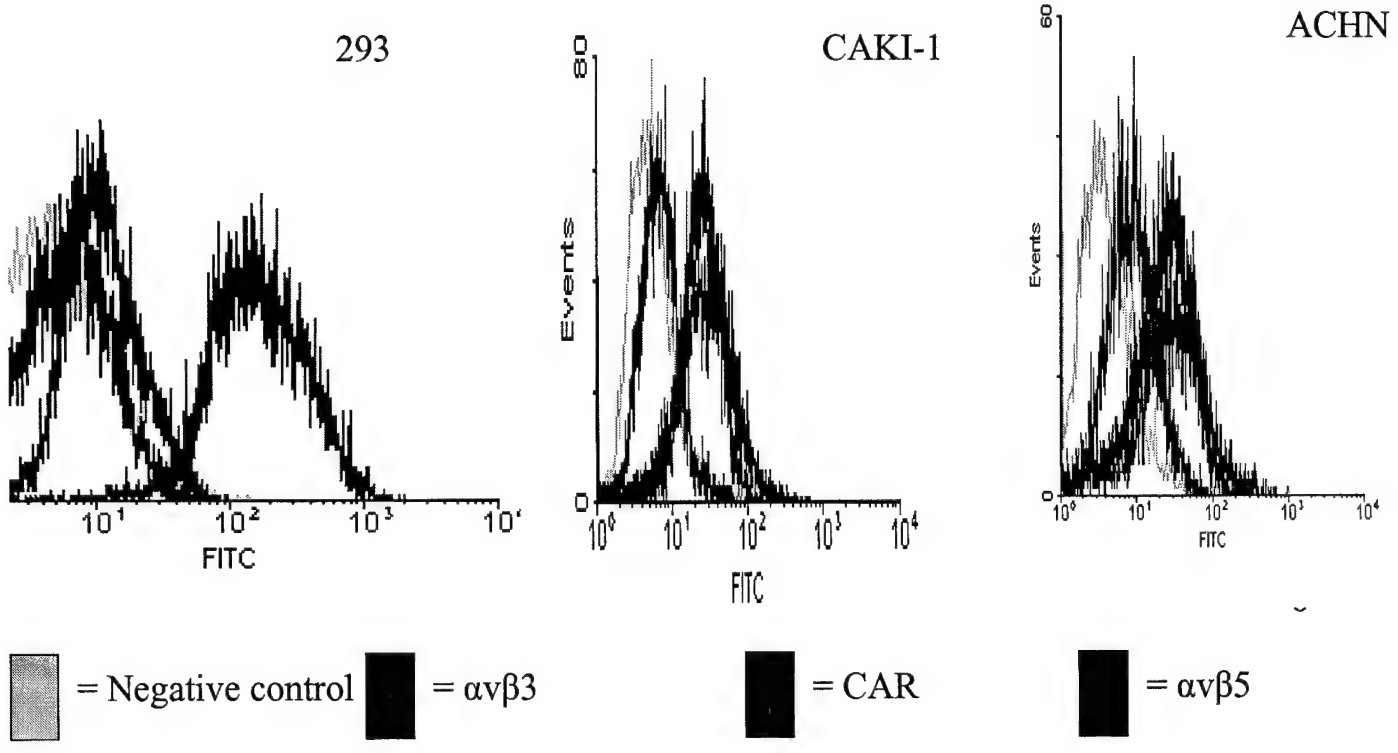

293
CAKI-1
ACHN
Events
FITC
= Negative control
=Ad5 knob
=Ad3 knob

B


293
CAKI-1
ACHN
Events
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= Negative control
= αvβ3
= CAR
= αvβ5

APPENDIX C

DATA:

Cox-2 Promoter Function in Prostate Cancer Cells

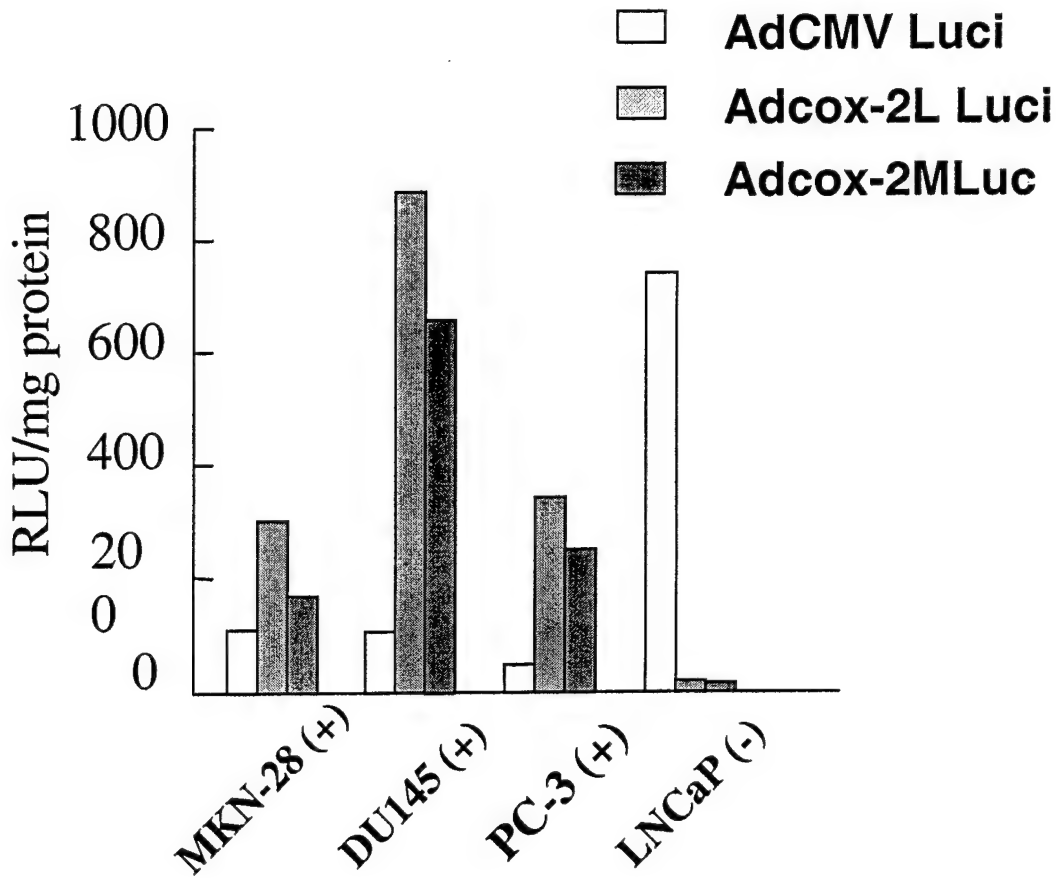
Plate 50000 cells / well

↓ 24 hr

Infect at 50 MOI

↓ 48 hr

Analyze
(luciferase
and protein assay)



APPENDIX D

[CANCER RESEARCH 61, 7882–7888, November 1, 2001]

A Midkine Promoter-based Conditionally Replicative Adenovirus for Treatment of Pediatric Solid Tumors and Bone Marrow Tumor Purging[1]

Yasuo Adachi, Paul N. Reynolds, Masato Yamamoto, Minghui Wang, Koichi Takayama, Shyuichiro Matsubara, Takashi Muramatsu, and David T. Curiel[2]

The Division of Human Gene Therapy, Departments of Medicine, Surgery, and Pathology, University of Alabama at Birmingham, Birmingham, Alabama 35294 [Y. A., P. N. R., M. Y., W. M., K. T., D. T. C.]; Department of Biochemistry, Faculty of Medicine, Kagoshima University, Kagoshima 890-8506, Japan [S. M.]; and Department of Biochemistry, Nagoya University School of Medicine, Nagoya 446-8550, Japan [T. M.]

ABSTRACT

The treatment of advanced neuroblastoma (NB) or Ewing's sarcoma (ES) is one of the major challenges in pediatric oncology. Both malignancies are refractory to conventional therapies and have an extremely poor prognosis. High-dose myeloablative radiochemotherapy with autologous bone marrow or peripheral blood stem cell rescue is one of the most aggressive treatments attempted for these diseases but is often undermined by residual tumor cells contaminating the graft. Thus, in this approach, purging of tumor cells from the graft is key to the prevention of relapse after transplantation. We investigated a novel approach to eliminate tumor cells from the bone marrow or peripheral blood stem cell graft without causing stem cell damage through the use of a conditionally replicative adenovirus (Ad). ES and NB are sensitive to Ad infection, and advanced NBs express a high level of the growth/differentiation factor midkine (MK). We confirmed in this study that ES cell lines (SK-ES-1 and RD-ES) are also sensitive to Ad infection and express high levels of MK. In contrast, CD34$^+$ stem cells are refractory to Ad infection and express very little MK. A conditionally replicative Ad in which the expression of E1 is controlled by the MK promoter achieved good levels of viral replication in NB or ES and induced remarkable tumor cell killing. On the other hand, this virus caused no damage to CD34$^+$ cells even after 3 h of infection at a dose of 1000 multiplicity of infection. We concluded that application of this replication-competent Ad to hematopoietic grafts could be a simple but effective procedure to achieve complete tumor cell purging.

INTRODUCTION

The development of CRAds[3] designed to replicate exclusively in tumor cells has the potential to significantly advance the treatment of malignancy (1, 2). Two basic strategies have been used, each of which involves alterations in the expression of genes essential for Ad replication. The first approach takes advantage of the disordered cell cycle regulation in tumor cells. This dysregulation means that certain viral genes, which have cell cycle regulatory functions, become dispensable in tumor cells, for example the genes responsible for activating the cell cycle by blocking Rb protein or p53. Thus, these genes have been completely or partially deleted with the aim of rendering the viruses incapable of replicating in normal cells (3). AdΔ24 or *dl*1520 (ONYX-015; Refs. 4, 5) are examples of this approach. The second strategy is the replacement of Ad promoters with tumor-specific promoters to drive the expression of genes essential for Ad replication. CRAds controlled by α-fetoprotein (6) or prostate-specific antigen promoters (7) have already been applied to the treatment of hepatomas or prostate cancers, respectively.

MK is a heparin-binding growth factor identified as a product of a retinoic acid-responsive gene (8, 9). The effects of MK include a neurotrophic function, mitogenic activity in fibroblasts (10), antiapoptotic activity (11), migration-promoting activity (12), angiogenic activity (13), enhanced plasminogen activator activity (14), and oncogenic transformation of fibroblasts (15). Of note, a close correlation between MK overexpression and tumorigenesis has been reported (16). The high expression level of MK observed in Wilms' tumors has lead to the recognition that MK is one of the target genes for the Wilms' tumor suppressor gene (WT1; Ref. 17). Furthermore, it has been reported that many kinds of malignant tumors express a high level of MK, for example, NBs (18), bladder carcinomas (19), lung cancers (20), breast cancers (21), pancreatic cancers (22), esophageal cancers (23), and gastrointestinal cancers (24, 25). In the case of NB, Nakagawara *et al.* (18) reported that the MK is overexpressed in primary tumors as well as in cell lines, and strong expression of MK is correlated with poor prognosis. On the other hand, MK status in ES is still under investigation. However, MK expression is not observed in mouse or human liver (10, 16), which is important in view of natural tropism of Ad for the normal liver. We established previously that the MK promoter could be used in an Ad vector where it retained the desirable features of high tumor activity and minimal activity in the liver, which enabled the mitigation of hepatic toxicity in a suicide gene approach to cancer therapy (26).

The treatment of advanced NB or ES is one of the major challenges in pediatric oncology. Because both of these pediatric malignancies are refractory to conventional therapy and have an extremely poor prognosis, high dose myeloablative radiochemotherapy with autologous stem cell transplantation has been attempted (27–30). However, tumor cell contamination of the autologous hematopoietic graft is a significant clinical problem leading to recurrence after transplantation (31, 32). Although peripheral blood stem cell transplantation or immunomagnetic CD34$^+$ selection (33, 34) has been used to deplete tumor cells, additional improvements in purging strategies are needed. Recently, investigators reported attempts to improve the efficacy of tumor cell purging in the case of breast cancers (35, 36) or multiple myelomas (37) through the use of replication-defective Ad vectors in a suicide gene approach or p53 antioncogene delivery. The rationale of using Ad vectors for the purging is that Ad shows high infection efficiency to tumor cells and low infectivity to hematopoietic cells. However, it is still questionable as to whether these anticancer strategies can transduce therapeutic genes to NB or ES cells with sufficient efficacy for an optimal therapeutic effect. This limitation is of particular importance in these tumor types, which have less cell-to-cell contact (and therefore less bystander effect; Ref. 38) and less abnormality of p53 (39, 40) compared with adult cancers. Thus, we hypothesized that a CRAd might overcome these limitations and deplete contaminating tumor cells more completely. Therefore, we investi-

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[3] The abbreviations used are: CRAd, conditionally replicative adenovirus; NB, neuroblastoma; ES, Ewing's sarcoma; Ad, adenovirus; MK, midkine; RT-PCR, reverse transcription-PCR; CMV, cytomegalovirus; MOI, multiplicity of infection; FCS, fetal calf serum; Adwt, wild-type Ad; rh, recombinant human; IL, interleukin; LITR, left inverted terminal repeat.

gated the application of a MK promoter-based, replication-competent Ad for the treatment of these MK-positive tumors using a model system relevant to the consideration of stem cell purging for advanced NB or ES. First, we evaluated the MK expression level of the target tumors or $CD34^+$ hematopoietic stem cells, then, based on these results, we assessed the efficacy of a CRAd in which expression of the essential *E1* gene is driven by the MK promoter. Thus, this strategy effectively takes advantage of natural Ad tropism plus transcriptional control to achieve a highly synergistic improvement in the specificity of target cell killing.

MATERIALS AND METHODS

Cells and Cell Culture. The Wilms' tumor G-401, NBY SK-N-SH, ES SK-ES-1 and RD-ES, and Burkitt's lymphoma Daudi cell lines were purchased from the American Type Culture Collection (Manassas, VA) and cultured in the medium recommended by the manufacturer. The melanoma MeWo cell line was a kind gift of Prof. Ian. R. Hart (St. Thomas Hospital, London, United Kingdom) and maintained in DMEM with 10% FCS. The 911 cell line (41), an E1 transcomplementing helper cell line that allows for the replication of E1-deleted Ad vectors, was obtained from Dr. Alex J. van der Eb, University of Leiden, The Netherlands. These cells were maintained in DMEM with 10% FCS and used for initial virus generation and propagation. All of the medium and FCS used in this study was purchased from Mediatech/Cellgro (Herndon, VA).

RNA Preparation and RT-PCR. Total cellular RNA of tumor cells or $CD34^+$ cells was extracted from 10^7 or 2×10^5 cells, respectively, using an RNeasy kit (Qiagen). GeneAmp RNA PCR core kit (Applied Biosystems) was available for cDNA synthesis and PCR amplification of cDNA products. Oligonucleotides corresponding to the sense strand of human MK cDNA (5′-ATGCAGCACCGAGGCTTCCT-3′: 1–20), the antisense strand of MK cDNA (5′-ATCCAGGCTTGGCGTCTAGT-3′: 450–428; 22), in the sense strand of human glyceraldehyde-3-phosphate dehydrogenase cDNA (5′-TCCCATCACCATCTTCCA-3′: 276–293), and in the antisense strand of glyceraldehyde-3-phosphate dehydrogenase cDNA (5′-CATCACGCCACAGTTTCC-3′: 638–655; 22, 42) were synthesized and used as primers for PCR. The PCR primers for MK transcript detection can discriminate MK transcripts from the genomic DNA. Total RNA (500 ng) of tumor cells or 50 ng of RNA of $CD34^+$ cells were applied to a standard RT-PCR protocol. The PCR conditions were as follows: 20 cycles of denaturation (95°C, 1 min), annealing (58°C, 1 min), and extension (72°C, 1 min), for tumor samples or 27 cycles of the same PCR profile for $CD34^+$ cell samples. PCR products were detected by 1% agarose gel electrophoresis with ethidium bromide staining.

Viruses and Viral Techniques. The replication-competent Ad AdMKE1, including the adenoviral E1A region under the control of human MK promoter containing 27 bp of exon 1 and 2285 bp of the 5′ flanking region of the human *MK* gene (43), was constructed using the "AdEasy" method (44) with incorporation of E1. The MK promoter we chose was too long to insert into an Ad genome without deletion of the adenoviral E3 region. Therefore, we constructed AdMKE1 with an E3 deletion by using the plasmid pAdEasy1 as the backbone for homologous recombination. Briefly, the multicloning site and right arm in pShuttle were replaced by MK promoter and the consecutive adenoviral E1 region extending from position 489 to 5789 of the Ad genome. The resultant shuttle vector was linearized with *Pme*I digestion and subsequently cotransfected into *Escherichia coli* BJ5183 with pAdEasy-1 Ad backbone plasmid. The recombinants were linearized with *Pac*I digestion and transfected into the E1 transcomplementing 911 cell line to generate AdMKE1. The replication competent Ad AdMKE1 was propagated in 911 cells and purified by double CsCl density centrifugation. We also constructed AdCMVE1, replacing MK promoter in AdMKE1 by CMV enhancer/promoter, as a control vector. The recombinant Ad vectors AdMKLuc and AdCMVLuc, encoding firefly luciferase gene under the control of human MK promoter or CMV enhancer/promoter, respectively, have been described previously (26). Adwt was used as the replication-competent control. Virus titers were determined by plaque assay in 911 cells.

Luciferase Assay. Tumor cells were plated in 24-well plates in triplicate at the density of 50,000/well. The next day, the cells were infected with AdMKLuc or AdCMVLuc at the MOI of 50 in DMEM with 2% FCS for 1 h and then maintained in complete medium. The infected cells were harvested and treated with 100 μl of lysis buffer (Promega, cat #E153A) after 2 days culture. A luciferase assay (Luciferase Assay System; Promega) and a FB12 luminometer (Zylux Corporation) were used for the evaluation of luciferase activities of Ad-infected cells. Luciferase activities were normalized by the protein concentration in cell lysate (Bio-Rad DC Protein Assay kit).

Assessment of Viral DNA Replication. Tumor cells were plated in six-well plates in triplicate at the density of 300,000/well. After overnight culture, cells were infected with replication-competent Ads (AdMKE1, AdCMVE1, and Adwt) or nonreplication Ad (AdMKLuc) at the MOI of 1 for 3 h and then cultured for 24 h. The harvest of infected cells was followed by viral DNA isolation using Blood DNA kit (Qiagen). Viral DNA was eluted with 100 μl of elution buffer [10 mM Tris Cl (pH 8.5)]. Eluted samples (1μl) were analyzed by real-time PCR analysis to evaluate Ad E4 copy number using a LightCycler (Roche). Oligonucleotides corresponding to the sense strand of Ad E4 region (5′-TGACACGCATACTCGGAGCTA-3′: 34885–34905), the antisense strand of E4 region (5′-TTTGAGCAGCACCTTGCATT-3′: 34977–34958), and TaqMan probe (5′-CGCCGCCCATGCAACAAGCTT-3′: 34930–34951) were synthesized, used as primers, and probed for real-time PCR analysis. The PCR conditions were as follows: 35 cycles of denaturation (94°C, 20 s), annealing (55°C, 20 s), and extension (72°C, 30 s). Ad backbone vector pTG3602 (Ref. 45; Transgene, Strasbourg, France) was available for making a standard curve for Ad E4 DNA copy number. E4 copy numbers were normalized by the β-actin DNA copy numbers.

MTS Assay. Tumor cells were plated in 96-well plates in quintuplicate at the density of 1000/well (ES cell lines, 3000/well). After overnight culture, cells were infected with nonreplication or replication-competent Ads at various MOI for 3 h. The infecting medium was then replaced with DMEM containing 5% FCS. Viable cells using MTS assay (CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay; Promega) were evaluated at the day when total cell death was microscopically observed in the wells of 1000 MOI of Ad5wt infection. The MTS color development was analyzed by an EL 800 Universal Microplate Reader (Biotec Instruments Inc.). In addition, similar experiments were conducted in which the number of viable cells present was determined by trypan blue staining and counting using a hemocytometer.

Soft Agar Assay. Tumor cells were plated in 24-well plates in triplicate at the density of 50,000/well. After overnight culture, cells were infected with AdMKLuc, AdMKE1, AdCMVE1, or Adwt at the MOI of 20 or 100 in DMEM containing 2% FCS. After 3-h virus infection, cells were dispersed and then plated by layering cell suspension (G-401 and MeWo, 3000/1.5 ml; SK-ES-1 and RD-ES, 5,000/1.5 ml) in 0.4% SeaPlaque agarose (BioWhittaker Molecular Applications) on six-well plates containing 2 ml of 0.53% bottom agarose. Agarose layers contained DMEM supplemented with 10% FCS. The number of colonies was enumerated after 10–14-day culture.

Hematopoietic Colony-forming Assays. Bone marrow $CD34^+$ cells were purchased from Clonetics. Cells were plated in 96-well plates in triplicate at a density of 5000/well. Cells were infected with AdMKLuc, AdMKE1, AdCMVE1, or Adwt at the MOI of 1000 for 3 h in 50 μl of Origen giant cell tumor conditioned medium (International, Inc.). After infection, 1000 cells were plated in Methocult GF H4434 (0.9% methylcellulose in Iscove's modified Dulbecco's medium, 30% fetal bovine serum, 1% BSA, 3 units/ml of rh erythropoietin, 100 μmol/liter 2-mercaptoethanol, 2 mmol/liter L-glutamine, 50 ng/ml rh stem cell factor, 10 ng/ml rh granulocyte macrophage colony-stimulating factor, and 10 ng/ml rh IL-3; StemCell Technologies Inc.). We counted hematopoietic colonies with >20 cells after 8 days of culture.

RESULTS

NB and ES Express High Level of MK. We wished to develop a strategy for the therapy of MK-positive pediatric tumors based on the use of a CRAd in which the MK promoter controls the expression of E1 (Fig. 1). *In vitro*, we first investigated a panel of tumor lines for MK expression. Candidate lines were investigated using an RT-PCR method as reported previously. We chose the G-401 and Daudi cells as positive and negative controls, respectively, as well as the MeWo melanoma line as a candidate Ad-infectable but MK-negative line. G-401, SK-N-SH, and two ES cell lines showed a strong PCR band of 450 bp corresponding to the MK product. On the other hand, the band

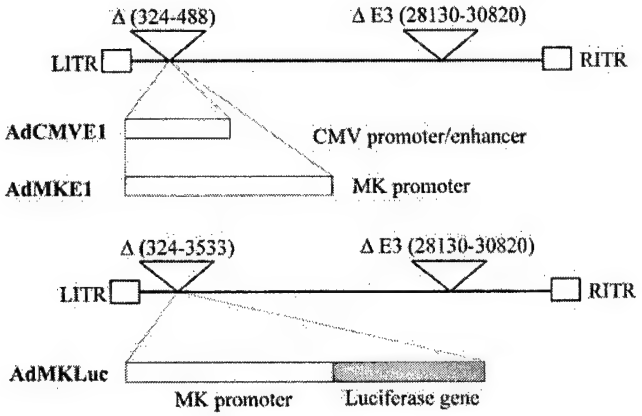

Fig. 1. Schema of the CRAd vectors used in this study. These vectors are constructed from E3 region-deleted Ad5 backbone and do not contain the Ad E1A promoter region expanding from nucleotide 324 to 488 of the Ad genome. Deletion of the E3 region was necessary, because the MK promoter we chose was too long to insert into the Ad genome without deletion of adenoviral E3 region. AdCMVE1 and AdMKE1 differ in the promoter driving E1A expression. Only AdMKLuc is the nonreplicating Ad control. We also use Adwt as a control.

obtained from the MeWo line was barely detectable (Fig. 2*A*). On the basis of this result, we proceeded with additional evaluation using G-401, SK-N-SH, SK-ES-1, and RE-ES as MK-positive tumor lines and MeWo as a MK-negative tumor line.

MK Promoter Retains Its Fidelity in the Ad Context. In the next step we assessed the activity of the MK promoter driving the expression of the luciferase reporter gene in an Ad vector (AdMKLuc) in the various cell lines (Fig. 2*B*). The data of G-401 and SK-N-SH are consistent with our previous report (26). Daudi cells are omitted because of their refractoriness to Ad infection. In all of the lines, luciferase expression was achieved using the positive control AdCMVLuc vector. In the MK-positive lines, the luciferase activity induced by AdMKLuc showed comparatively high activity, especially in ES cell lines. Even in the SK-N-SH line, which has the lowest MK/CMV ratio of the MK-positive lines, the luciferase activity induced by AdMKLuc was 12% of that induced by AdCMVLuc. In contrast, the MK/CMV ratio of MeWo was 1.5%. Thus, these data confirm that the MK promoter retains its fidelity in the Ad vector.

MK Promoter Driving CRAd Shows Replication Specificity in NB and ES. To exploit the cell specificity of the MK promoter in a CRAd context, we then constructed a recombinant Ad (AdMKE1) in which the native E1 promoter is replaced with the MK promoter. The genomic structures of replication-competent Ads used in this study are depicted in Fig. 1. In addition to using the MK promoter to control E1 expression we also constructed a control vector (AdCMVE1) in which E1 expression is controlled by the CMV promoter. These viruses are deleted in the E3 region extending from 28130 to 30820 Ad5 nucleotides and the E1A promoter region extending from 342 to 488 Ad5 nucleotides. The deleted E1A promoter region, containing native E1A TATA box, was replaced with either the MK promoter or CMV enhancer/promoter to produce the viruses AdMKE1 or AdCMVE1, respectively.

To determine the specificity of replication of the AdMKE1, we infected the panel of MK-positive and negative lines and then used quantitative real-time PCR to determine the level of amplification of viral DNA. The nonreplicative AdMKLuc vector and wild-type Ad5 virus were used as negative and positive controls, respectively. We found that the rate of increase in DNA copy number for the AdMKE1 virus was higher than that of Adwt in every MK-positive cell line. In contrast, the copy number of AdMKE1 was 10 times less than that of Adwt in MeWo MK-negative cells (Fig. 3). In addition, the replication of AdMKE1 was relatively much less than the replication of a control-replicative Ad (AdCMVE1) in the MK-negative MeWo line than in the MK-positive lines. These results indicate that the MK promoter retains fidelity even in the replication-competent Ad.

Specific Cell Killing Efficacy of MK Promoter Driving CRAd. We next investigated the ability of AdMKE1 to achieve cell killing in the MK-positive lines using MTS assay. In this assay, the number of viable cells is estimated based on the conversion of MTS into soluble formazan, a process accomplished by dehydrogenase enzymes. Because this enzyme activity is related to metabolic activity of the cells, it could be influenced by the process of Ad replication *per se*. However, despite this caveat, the profiles of the viral dose-response curves allowed assessment of the effect of replication-competent Ad infection on cell viability, and as an additional validation, studies were conducted in which cell number was determined by trypan blue exclusion and cell counting, which gave similar results (data not shown). For all of the MK positive lines, AdMKE1 had much better

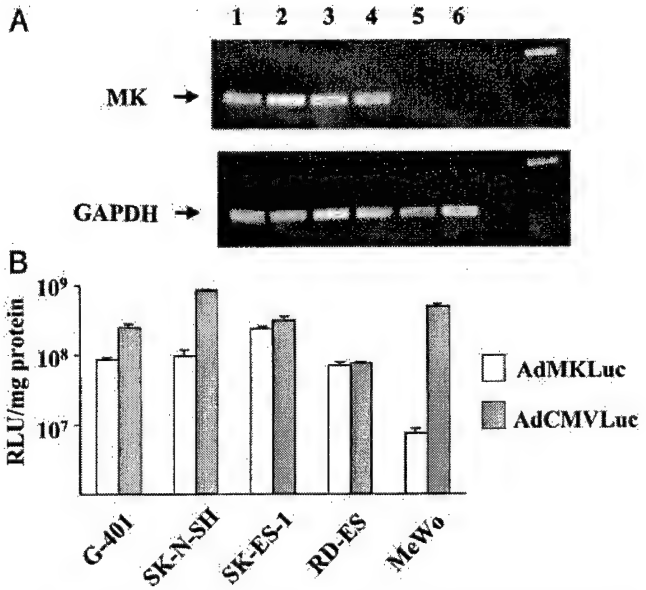

Fig. 2. *A*, evaluation of MK expression of the tumor cells. *Lane 1*, G-401 (Wilms' tumor); *Lane 2*, SK-N-SH (NB); *Lane 3*, SK-ES-1 (ES); *Lane 4*, RD-ES (ES); *Lane 5*, MeWo (melanoma); *Lane 6*, Daudi cells (lymphoma). G-401 and Daudi cells are reported MK-positive and negative respectively. *B*, luciferase expression in cell lines. Infection of AdMKLuc or AdCMVLuc at a MOI of 50. The ratios of luciferase activity induced by AdMKLuc compared with AdCMVLuc (MK:CMV) are as follows: G-401, 0.35; SK-N-SH, 0.12; SK-ES-1, 0.75; RD-ES, 0.95; and MeWo, 0.015; *bars*, ± SE.

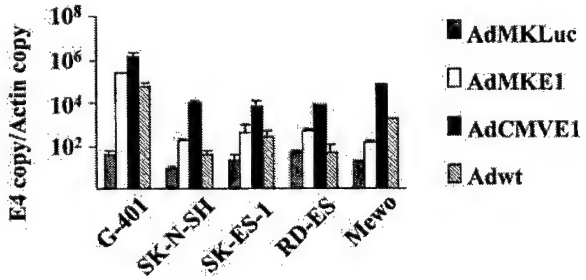

Fig. 3. Assessment of viral DNA replication 24 h after infection. Because all of the vectors tested here contained complete E4 region, the viral DNA replication rate was evaluated by measuring the E4 copy number using the quantitative real-time PCR method. The nonreplicative AdMKLuc vector and Adwt virus were used as negative and positive controls, respectively; *bars*, ± SE.

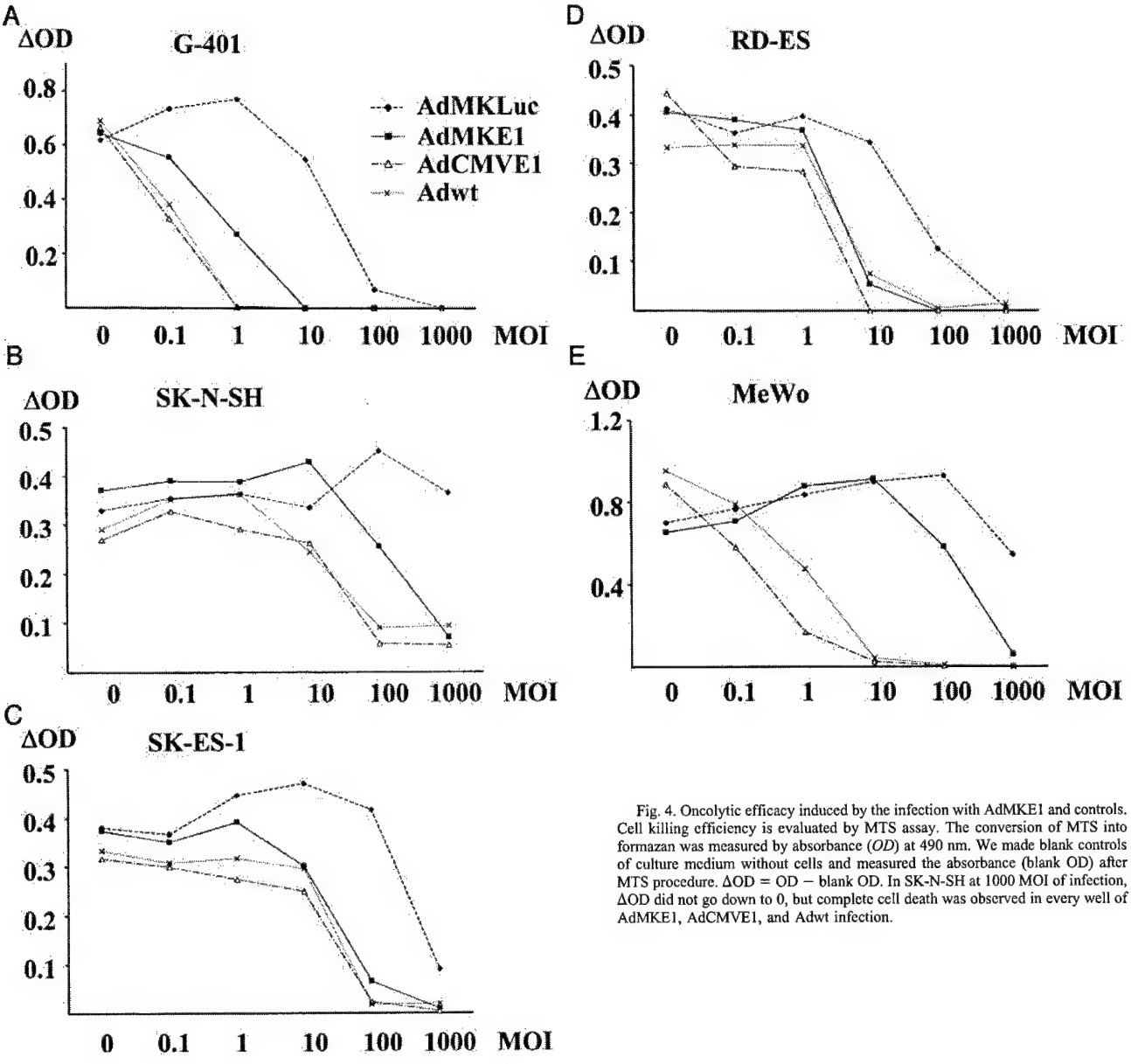

Fig. 4. Oncolytic efficacy induced by the infection with AdMKE1 and controls. Cell killing efficiency is evaluated by MTS assay. The conversion of MTS into formazan was measured by absorbance (*OD*) at 490 nm. We made blank controls of culture medium without cells and measured the absorbance (blank OD) after MTS procedure. ΔOD = OD − blank OD. In SK-N-SH at 1000 MOI of infection, ΔOD did not go down to 0, but complete cell death was observed in every well of AdMKE1, AdCMVE1, and Adwt infection.

cell-killing efficacy than the MK-negative MeWo line (Fig. 4). Furthermore, in ES cell lines, the effectiveness of AdMKE1 was comparable with AdCMVE1 or wild-type Ad5, as reflected by the similar profile of dose-response curves (Fig. 4, *C* and *D*). In aggregate these data demonstrated that AdMKE1 achieved potent, selective cell killing in the MK-positive lines.

To determine the capability of the purging strategy to accomplish eradication of clonogenic MK-positive tumors, treatment of these cells with AdMKLuc, AdMKE1, AdCMVE1, or wild-type Ad5, was followed by analysis for soft agar colony formation (Table 1). The SK-N-SH line was omitted, because it was not able to achieve colony formation in this assay *per se*. The data obtained by soft agar assay correlated well with the results of MTS assay. In MK-positive cells, the efficacy of eradicating tumor formation by AdMKE1 was comparable with that by AdCMVE1 or wild-type Ad5. It is noteworthy that almost complete prevention of colony formation was achieved in G-401 or RD-ES cells when the inoculum was infected with AdMKE1 at an MOI of 100 for 3 h. On the other hand, in the MeWo cells, efficacy of AdMKE1 seemed to be much less compared with AdCMVE1 or wild-type Ad5. Thus, these results were encouraging in regard to our aim of developing a purging strategy for MK-positive tumors.

Table 1 *Colony Formation in Soft Agar*[a]

	MK (+)			MK (−)
	G-401	RD-ES	SK-ES-1	MeWo
Mock	93.7 (15.9)	148.0 (23.6)	329.7 (79.8)	242.0 (59.4)
MOI 20				
AdMKLuc	82.0 (7.8)	85.3 (28.2)	182.3 (20.3)	228.7 (32.8)
AdMKE1	1.3 (0.58)	63.7 (21.4)	58.3 (22.7)	173.7 (39.6)
AdCMVE1	0	3.3 (2.5)	60.7 (13.4)	1.7 (1.5)
Adwt	0	41.7 (6.0)	24.7 (16.7)	7.0 (2.0)
MOI 100				
AdMKLuc	16.3 (1.5)	34.0 (11.3)	116.7 (24.7)	67.3 (25.1)
AdMKE1	0.7 (0.58)	0.3 (0.58)	20.0 (12.5)	30.0 (7.9)
AdCMVE1	0	0	10.7 (5.5)	0
Adwt	0	0	5.3 (3.1)	0

[a] Data are means (±SD) of triplicate determinations.

MK Promoter Driving CRAd Confers No Damage to Hematopoietic Stem Cells. Having shown evidence of efficacy for the AdMKE1 approach for MK-positive tumors, the final step was to evaluate the potential toxicity of this approach for hematopoietic stem cells. The MK expression level in $CD34^+$ cells was evaluated by RT-PCR as described before. $CD34^+$ cells express very low levels of MK compared with G-401 (Fig. 5*A*). To investigate the potential toxicity of AdMKE1 infection on hematopoietic stem cells, we treated $CD34^+$ cells with 1000 MOI of AdMKLuc, AdMKE1, AdCMVE1, or Adwt for 3 h and then cultured for 8 days (Fig. 5*B*). Compared with untreated (Mock) or nonreplication Ad-infected group (AdMKLuc), no reduction of hematopoietic colonies was observed in the group infected with AdMKE1. On the other hand, significant reduction of hematopoietic colonies was confirmed in the group infected with AdCMVE1 or wild-type Ad5. Compared with tumor cell lines tested in this study, $CD34^+$ cells were actually less susceptible to the cell killing effect of AdCMVE1 or Adwt attributable to the poor Ad infectability of hematopoietic stem cells. Nevertheless, at high doses, toxicity was observed with the nonselective control viruses. Thus, the incorporation of the MK promoter into the CRAd strategy achieved a higher level of safety without compromising the basic efficacy against tumor cells. These data support our hypothesis that AdMKE1 would be a good candidate for bone marrow purging for NB or ES patients based on the combined attributes of Ad tropism as well as the transcriptional specificity of the MK promoter.

DISCUSSION

Conditionally replicative Ads, which show tumor specific replication and oncolysis, are promising new therapies for malignancies resistant to conventional treatments. In the current report, we demonstrate a strategy based on the use of a replication-competent Ad controlled by a tumor-specific promoter relevant to incurable pediatric solid tumors. Concurrently, we demonstrated that use of this replication-competent virus avoided causing damage to hematopoietic stem cells by virtue of both the low tropism of the virus for the cells as well as the low activity of the MK promoter in these cells.

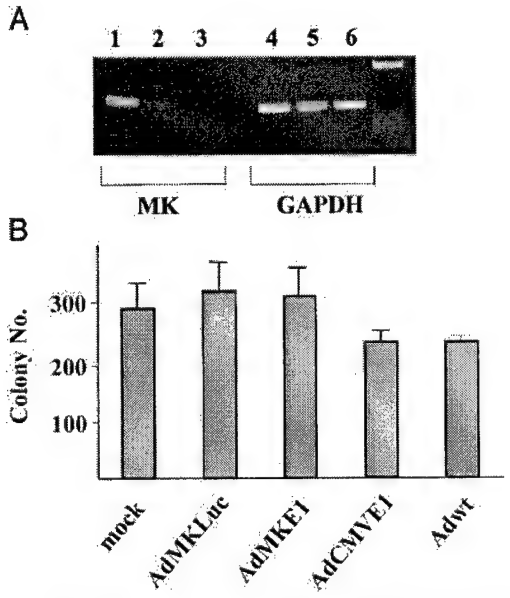

Fig. 5. *A*, evaluation of MK expression level in $CD34^+$ cells. Total RNA (50 ng) was analyzed using RT-PCR. *Lanes 1* and *4*, G-401; *Lanes 2* and *5*, $CD34^+$ hematopoietic cells; *Lanes 3* and *6*, Daudi cells. Very scant bands corresponding to MK transcripts were observed in $CD34^+$ and Daudi cell samples. *B*, hematopoietic colony formation derived from $CD34^+$ cells infected with Ad vectors. Hematopoietic colonies were counted after 8 days of culture in Methocult GF H4434 (0.9% methylcellulose in Iscove's modified Dulbecco's medium, 30% fetal bovine serum, 1% BSA, 3 units/ml of rh erythropoietin, 100 μmol/l 2-mercaptoethanol, 2 mmol/l L-glutamine, 50 ng/ml rh stem cell factor, 10 ng/ml rh granulocyte macrophage colony-stimulating factor, and 10 ng/ml rh IL-3). Data are means of triplicate determinations; *bars*, ± SE.

The treatment of NB and ES is an area in which novel therapies are urgently needed. To our knowledge, this is the first report to address the question of using a CRAd approach for bone marrow purging. In addition, the method we presented herein, which involved only 3-h incubation of hematopoietic graft with CRAd, should be easily applicable to standard bone marrow transplantation protocols. Despite the fact that advanced NB and ES may show good initial responses to radiochemotherapy, aggressive recurrence frequently leads to the tragic loss of young lives. High-dose chemotherapy with autologous stem cell rescue has been introduced in an effort to overcome the high mortality caused by these devastating pediatric tumors (27–30). In this regard, complete removal of tumor cells contaminating the autologous hematopoietic graft has been a challenging problem but is essential for optimal outcome. Recently, several attempts at hematopoietic stem cell purging using nonreplicative Ad vectors have been reported, aimed at the treatment of breast cancer (35, 36), multiple myeloma (37), and prostate cancer (46). These approaches may be limited by the same issues that have compromised the direct application of Ad vectors to solid tumors, that is, poor penetration of the vector into the tumor mass. In this regard, the tumor cells in the bone marrow samples aspirated from tumor patients form clusters and cannot be expected to be effectively transduced by conventional replication-incompetent Ad. We speculate that the clusters of tumor cells justify the development of a CRAd approach to purging, allowing for improved intratumoral spread and penetration. Furthermore, it should be noted that NB and ES have fewer gap junctions (38) and less abnormalities of p53 (39, 40) compared with adult malignancies, thus making them less susceptible to the use of herpes simplex virus thymidine kinase (HSVTK) or p53 gene therapy approaches. We believe that the bone marrow purging of MK-positive pediatric solid tumors using AdMKE1 may be an ideal strategy, having the benefits of intratumor spread, and transductional and transcriptional specificity.

Lack of toxicity for hematopoietic cells is a key property for any Ad-based bone marrow purging strategy. However, prevention of hepatic toxicity is also another important aspect (47, 48) even in *ex vivo* strategies. Tumor cells infected with replication-competent Ad may release new viruses *in vivo*. Should this occur, there would be a potential for *in vivo* toxicity, especially in the liver, because this is the predominant site of Ad vector localization after systemic injection. In this regard, we reported previously the potential benefits of using MK promoter for cancer gene therapy based on the ability to avoid hepatic injury attributable to the very low level of promoter activity in this organ (26). Because AdMKE1 showed specificity in both replication rate and cytotoxicity as shown in MK-positive and negative cells, it can be expected to render less toxicity to the liver compared with AdCMVE1 or wild-type Ad5. However, despite the selectivity of AdMKE1 for replication in MK-negative cells, the degree of control is not absolute, because some toxicity could be seen even in the MK-negative MeWo cells. We believe this arises as a result of a low level of expression of E1 attributable to the promoter activity of the LITR and/or encapsidation signal. Thus, additional improvements in specificity could be achieved before clinical application. These approaches could entail the use of insulator sequences between the LITR and MK promoter (49) or by placing other essential viral genes in addition to E1 under MK promoter control (*e.g.*, E4). Nevertheless, the degree of toxicity we observed in the MK-negative line was significantly less than that for the positive lines. At this time, the

correlation between toxicity in putative negative cells in *in vitro* assays and the degree of control needed to prevent damage to normal tissues *in vivo* is uncertain. Replicative viruses already in clinical trial have shown at least some low level of viral production and/or toxicity in negative cell lines *in vitro* (7, 50–54). Unfortunately, at this time, no suitable animal models exist for the assessment of CRAd toxicity *in vivo*.

The application of a MK-promoter based CRAd for NB or ES therapy may have utility beyond stem cell purging. Clinical trials of immunogene therapy for NB based on Ad gene transduction have been reported (55). In this study the authors used irradiated autologous NB cells transduced with the gene for IL-2 as an immunostimulatory agent. However, a new trial using unirradiated autologous cells has been proposed to maximize immunogenicity. The protocol seeks to use unirradiated cells as the vaccine, thus raising safety concerns (56). It might be plausible to coinfect cells with both the MK CRAd and a vector carrying an immunostimulatory *IL-2* gene, thus ensuring the eventual clearance of the injected cells but also potentially improving overall immunostimulatory effect. The potential application of a CRAd to tumor vaccine strategies is an area that warrants additional investigation. In this regard, most of the malignancies refractory to conventional treatments are MK-positive tumors (18–25), thus, a MK promoter-based approach would be rational.

We believe that the data presented here provides a basis for the additional development of replication-competent Ad strategies based on the MK promoter for the therapy of pediatric and adult cancers.

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APPENDIX E

Deletion of E1B 19k improves the efficacy of a midkine-promoter based conditionally replicative adenovirus for neuroblastoma cells[1]

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Running title: Replicating adenoviral vector controlled by midkine promoter

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[3]The abbreviations used are; NB, neuroblastoma; Ad, adenovirus; MK, midkine; CRAd, conditionally replicative adenovirus; RT, reverse transcriptase; PCR, polymerase chain reaction; GAPDH, glyceraldehydes 3-phosphate dehydrogenase CMV, cytomegalovirus; MOI, multiplicity of infection; DMEM, Dulbecco's Modification of Eagle's Medium; FCS, fetal calf serum; PBS, phosphate buffer saline; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium: tsp, tumor specific promoter.

Abstract

Conditionally replicative adenoviruses (CRAds) are being considered as novel therapies for a variety of malignancies, and several clinical trials have been undertaken. Since neuroblastomas (NBs) are sensitive to adenoviral infection, and advanced NB is refractory to conventional therapy, we have evaluated the use of CRAds for this disease. We previously constructed a replication competent adenovirus (AdMKE1) that achieves tumor-specific replication by virtue of the replacement of the native viral E1 promoter with the tumor specific midkine (MK) promoter. This vector was shown to have utility in an in vitro bone marrow tumor cell purging model. However, clinical trials are revealing that the efficacy of first generation CRAds is suboptimal, and that further modifications or complementary approaches may be necessary. In this regard, it has been reported that replication competent Ad with a deletion of the E1B 19kD gene achieves more effective oncolysis than vectors with intact E1. Thus, we constructed a new virus, AdMKE1(19kdel), in which the modified E1 region is under the control of the MK promoter, and evaluated the oncolytic effect and replication properties of this virus in neuroblastoma cells in vitro. AdMKE1(19kdel) showed a significant improvement in cell killing effect in the SK-N-SH NB cell line as compared to the original AdMKE1 virus, whilst retaining its cell killing specificity. This improvement was not related to the replication efficiency of this virus, but possibly due to the early cell burst and early virus release from the NB cells. Thus, the combination of the MK promoter and the E1B modification may ultimately lead to a more effective clinical agent.

Introduction

Advanced NB is refractory to conventional therapies and the treatment of this condition is one of the most challenging problems in pediatric oncology (1-3). We have been investigating a new strategy to treat advanced NB using conditionally replicative adenoviruses (CRAds) (4). CRAds are modified viruses designed to replicate exclusively in the tumor cells, thus achieving therapeutic cytolysis whilst being non-toxic to normal tissues (5-7). Several clinical trials using CRAds have been undertaken, and the early evidence suggests that while this approach is safe, the tumor cell killing efficacy is often suboptimal. Thus, further improvements in the basic strategy are required.

We previously established a novel virus (MK-CRAd) whose E1A transcription is controlled by tumor specific midkine (MK) promoter (4). MK is a heparin-binding growth factor (8,9) with properties including a neurotrophic function (10), anti-apoptotic activity (11), and angiogenic activity (12). The use of the MK promoter as the basis for oncolytic therapy for the NB is logical because MK is over-expressed in NBs and strong expression of MK is correlated with poor prognosis (13). Moreover, this promoter retains a desirable feature of minimal activity in the liver even in the Ad context (14). In the case of intravenous Ad vector injection, mitigation of liver toxicity is a key property, because the liver is the predominant site of Ad vector localization after systemic administration (14-16). Although the use of non-replicating Ad vectors carrying a suicide gene may be an effective approach (17,18), it is questionable as to whether these anticancer strategies can transduce therapeutic genes to NB with sufficient efficacy for an optimal therapeutic effect. This limitation is of particular importance in these tumor types, which have less

cell-to-cell contact than epithelial neoplasms, and therefore less bystander effect, which is an important factor in the efficacy of suicide gene therapy using the HSV-TK system (19). On the other hand, a CRAd strategy may circumvent the limitations of the loose cell contact observed in NB tumors. Furthermore, NB has less abnormality of p53 compared with adult cancers (20), which implies that NB may not be susceptible to Ad p53 therapy. Based on these rationales, we evaluated a MK-CRAd as a therapeutic agent for NB (4). However, the NB cell killing effect induced by the MK-CRAd infection was lower than that observed in the other MK-postive tumors tested (Ewing's sarcomas or Wilms' tumors). Some modifications of the MK-CRAd seemed to be required to achieve sufficient oncolysis of NBs. One such approach involves more extensive manipulation of the Ad genome, particularly with respect to the early genes. The Ad genome carries five early transcription units (E1A, E1B, E2, E3, and E4), two delayed early units, and one late unit. E1A expression activates every viral early gene promoter and plays an important role in the regulation of viral replication and propagation. Recently, it has been reported that a replicating Ad deleted for the E1B19k gene showed enhanced cell killing efficacy compared with a replicating Ad having an intact E1 region (21,22). This property may be related to earlier viral release from infected cells and improved cell to cell spread. Thus, we have modified the MK-CRAd by deleting E1B19k region from original MK-CRAd and evaluated the oncolytic efficiency of these vectors against NB SK-N-SH cells

Materials and Methods

Cells and cell culture

The NB SK-N-SH and Wilms' tumor G-401 were purchased from the American Type Culture Collection (ATCC) (Manassas, VA) and cultured in the medium recommended by the manufacturer. The melanoma MeWo cell line was a kind gift of Prof. Ian R. Hart (St. Thomas Hospital, London, United Kingdom). The 911 cell line (23), a human embryonic retinoblastoma line transformed with a plasmid containing E1 region of the Ad5 genome, was maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) and used for initial virus generation and propagation. All media and FCS using in this study were purchased from Mediatech/Cellgro (VA).

Viruses and Viral Techniques

The construction of the replication competent Ad AdMKE1, including adenoviral E1A region under the control of human MK promoter containing 27 bp of exon 1 and 2285 bp of the 5' flanking region of the human MK gene (24), was described in the previous report (4). A deletion of the adenoviral E3 region was required to insert the 2.3kb of the MK promoter into the Ad genome. To generate an E1B modified Ad, we made shuttle vectors containing E1 region with a deletion of E1B 19k coding region (1712-2000) using PCR methods. These shuttle vectors were then used for homologous recombination with an Ad backbone plasmid (pAdEasy1) (25). The recombinants were transfected into the E1 transcomplementing 911 cell line to generate AdMKE1(19kdel). These MK-CRAds were propagated in 911 cells and purified by double CsCl density centrifugation. We also constructed AdCMVE1, replacing MK promoter in AdMKE1 by CMV enhancer/promoter, as a control vector. The recombinant Ad vector AdMKLuc (14),

encoding firefly luciferase gene under the control of human MK promoter, was used as a replication deficient control. Wild-type Ad (Adwild) was used as a replication competent control. Virus titers (plaque forming units, pfu) were determined by counting the plaque formation in 911 cells 14 days after infection.

RNA preparation and Reverse Transcription of RNA and PCR amplification of cDNA (RT-PCR)

Total RNA of Ad infected tumor cells was extracted using an RNeasy kit (Qiagen). GeneAmp RNA PCR core kit (Applied Biosystems) was available for cDNA synthesis and PCR amplification of cDNA products. DNase 1 digestion followed by heat inactivation procedure was performed to avoid viral genome DNA contamination in the RNA samples. Oligonucleotides for primer to detect the transcripts of Ad E1A, E1B(19k), E1B(55k), and human glyceraldehydes 3-phosphate dehydrogenase (GAPDH) (26) are as follows, Ad E1A; sense strand (5'-ACGGTTGCAGGTCTTGTCATTATC-3': 1014-1038), antisense strand (5'-AAGCAAGTCCTCGATACATTCCA-3': 1494-1472): Ad E1B(19k); sense strand (5'-GCTTGGGAGTGTTTGGAAGATTT-3': 1720-1742), antisense strand (5'-CTGCTCCTCCGTCGGTATTAT-3': 2160-2140): Ad E1B(55k); sense strand (5'-GAATGAATGTTGTACAGGTGGCT-3': 2239-2261), antisense strand (5'-AGGAAAACCGTACCGCTAAAATTG-3': 2773-2750) (27): human GAPDH; sense strand (5'-TCCCATCACCATCTTCCA-3': 276-293), antisense strand (5'-CATCACGCCACAGTTTCC –3': 655-638). Although there are two kinds of transcripts (12S and 13S) originated from E1A region, the PCR primers for E1A can only detected the 13S transcript and cannot detect the 12S transcript. Five hundred ng of total RNA of

tumor cells were applied to a standard RT-PCR protocol. The PCR condition was 30 or 35 cycles of denaturation (95°C, 1 min); annealing (52°C, 1 min) and extension (72°C, 1 min). PCR products were detected by 1% agarose gel electrophoresis with ethidium bromide staining.

Real-time PCR (LightCycler, Roche) analysis combined with reverse transcriptase preparation was also used for the measurement of E1A transcripts. Oligonucleotides corresponding to the sense strand of Ad E1 region (5'-AACCAGTTGCCGTGAGAGTTG-3': 1433-1453), the antisense strand of E1 region (5'-CTCGTTAAGCAAGTCCTCGATACA-3': 1500-1476), TaqMan probe (5'-CACAGCCTGGCGACGCCCA-3': 1473-1455) (27) were synthesized and used as primers and probe for real-time PCR analysis. The PCR conditions were as follows: 35 cycles of denaturation (94°C, 20 sec), annealing (55°C, 20 sec) and extension (72°C, 30 sec). Ad backbone vector pTG3602 (Transgene, Strasbourg, France) was available for making a standard curve for Ad E1A DNA copy number. E1A copy numbers were normalized by the total RNA (ng).

Oncolysis assay

SK-N-SH or MeWo cells were plated in 12-well plates at the density of 2×10^5 /well or 1×10^5 /well respectively. After overnight culture, cells were infected with non-replication or replication-competent Ads at several MOIs (multiplicity of infection) for 3 hours. The infecting medium was then replaced with complete medium. Eight days after infection, the adhesion cells were washed gently with PBS (phosphate buffer saline) and

fixed with 10 % buffered formaldehyde, and then processed into the staining with 1 % crystal violet solution.

MTS assay was also available for the evaluation of oncolytic efficacy induced by CRAd infection. Tumor cells were plated in 96-well plates in triplicate at the density of 3000/well. After overnight culture, cells were infected with non-replication or replication competent Ads at various MOIs for 3 hours. The infecting medium was then replaced with complete media. Viable cells using MTS assay (CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay; Promega) were evaluated at 8 days after virus infection. The MTS color development was analyzed by an EL 800 Universal Microplate Reader (Biotec Instruments Inc.).

Assessment of viral DNA replication in NB cells

SK-N-SH cells were plated in 6-well plates in triplicate at the density of 300,000/well. After overnight culture, cells were infected with non-replication Ad or replication competent Ads at the MOI of 1 for 3 hours and then cultured in complete medium. The infected cells were harvested with their supernatant 1, 3, and 5 days after infection. The viruses were extracted from cell suspension by three times of freeze-thawing method. DNase 1 preparation was performed to remove the naked DNA floating in the virus solution. Then, EDTA, SDS, and proteinase K were added to the solutions (final concentration: EDTA, 20 mM; SDS, 0.5 %; proteinase K, 0.2 mg/ml) for inactivation of DNase 1 and isolation of encapsidated viral DNA. The mixtures were incubated at 52^0 C for 1 hour, and followed by phenol/chloroform extraction. The purified viral DNA was dissolved in distilled water. Real-time PCR analysis was available for evaluating Ad copy number. An Ad E4 copy number was evaluated as a viral replication, because all vectors

using in this study contained Ad E4 region. Oligonucleotides corresponding to the sense strand of Ad E4 region (5'-TGACACGCATACTCGGAGCTA-3': 34885-34905), the antisense strand of E4 region (5'-TTTGAGCAGCACCTTGCATT-3': 34977-34958), TaqMan probe (5'-CGCCGCCCATGCAACAAGCTT-3': 34930-34951) (4) were synthesized and used as primers and probe for real-time PCR analysis. The PCR conditions were as follows: 35 cycles of denaturation (94°C, 20 sec); annealing (55°C, 20 sec) and extension (72°C, 30 sec). The plasmid pTG3602 (containing the entire Ad genome) was available for making a standard curve for Ad E4 DNA copy number. We also evaluated encapsidated viral DNA copy number in the supernatant using the similar procedure.

Results

The Ads used in this study are depicted in figure 1. G-401 Wilms' tumor cells were chosen for the initial evaluation of these vectors, because this cell line is well characterized as a MK positive tumor and is highly sensitive to the Ad infection. We first assessed the profiles of Ad E1A, E1B19k, or E1B55k mRNA expression in G-401 cells after Ad vector infection using RT-PCR. PCR primers for E1A transcripts were designed to detect only 13S transcript in the E1A transcripts. The RT-PCR analysis showed that the cells infected with AdMKE1, AdCMVE1, or Adwild expressed all of E1 components. Although the cells infected with AdMKE1(19kdel) expressed the mRNA corresponding to E1B 55k, they did not express E1B 19k mRNA (Fig. 2).

Next, we investigated the E1A expression level in SK-N-SH cells infected with Ads (Fig. 3A). This experiment was repeated three times, and reproducible results were obtained. Real time RT-PCR analysis was also used for the measurement of E1A transcript levels (Fig. 3B). Previously, we reported that MK promoter activity was about 15 % of CMV activity in SK-N-SH cells in the context of Ad vectors carrying reporter genes (14). The difference of E1A expression between AdMKE1 and AdCMVE1 in this study was consistent with the previous data. E1A expression levels seen with the MK vectors were comparable to that seen with wild-type Ad. Thus, we confirmed the ability of the MK promoter to drive E1A expression in the neuroblastoma line.

To investigate the ability AdMKE1(19kdel) to achieve NB cell killing, crystal violet staining was used to visualize the surviving cells 8 day after infection. As compared with

AdMKE1, AdMKE1(19kdel) achieved a substantial improvement in oncolysis of SK-N-SH cells. It was notable that AdMKE1(19kdel) induced oncolysis more effectively than AdCMVE1 and its efficacy was comparable to that of Adwild (Fig. 4a). To assess the specificity of oncolytic efficacy induced by AdMKE1(19kdel), MeWo cells (MK negative melanoma cell line) were infected with the viruses and the cell killing efficiency was evaluated. The cell-killing effect in the MeWo cells induced by AdMKE1(19kdel) infection was insignificant and comparable with that induced by AdMKE1 which has been reported previously (4). In contrast, infection with AdCMVE1 or Adwild resulted in significant cell killing of MeWo cells (Fig. 4b). Thus, the 19k deletion had achieved an improvement in the cell killing effect for MK positive cells without any loss of specificity. These results were then confirmed using the more quantitative MTS assay. Eight days after virus infection, the advantage of AdMKE1(19kdel) compared to AdMKE1 for cell killing of SK-N-SH is clear (Fig 5A), as is the specificity shown by the relative lack of toxicity for the MK negative MeWo line (Fig 5B).

To examine the correlation between viral replication and oncolytic efficiency, viral DNA production was measured with the passage of time after virus infection (Fig. 6). Viral copy number in the cells infected with AdCMVE1 was about 100 times higher than that with AdMKE1 on day 1 after infection and about 10 times higher on day 3 and 5. Thus, the improved cell killing of AdCMVE1 vs AdMKE1 could be explained on the basis of faster viral replication. On the other hand, AdMKE1(19kdel) did not replicate more effectively than AdMKE1 in SK-N-SH cells, despite the fact that the cell killing effect of AdMKE1(19kdel) was significantly greater than AdMKE1 and was in fact comparable to

AdCMVE1. This would suggest that the efficacy gains seen with AdMKE1(19kdel) are not simply due to increased viral replication.

Previously, it was reported that an E1B-19kD mutant virus is released more rapidly from the tumor cells and that may lead to the improvement of the cell killing efficiency (21,22). Thus, we investigated whether AdMKE1(19kdel) might be released from NB cells earlier than other viruses after infection. SK-N-SH cells were plated in 6-well plates in triplicate at the density of 100,000/well. After overnight culture, cells were infected with Ads at the MOI of 10 for 3 hours and then cultured in complete medium. Supernatants were harvested 3 and 5 days after infection and the DNA copy number of encapsidated virus in the supernatants were measured (Fig. 7). Notably, virus DNA copy number in the supernatant of AdMKE1(19kdel) infection group was higher than that of AdMKE1 or AdCMVE1 at day 3. These data suggest that AdMKE1(19kdel) is released from NB cells earlier compared with other CRAds lacking the Ad E3 region. However, the amount of released virus from the NB cells infected with Adwild was comparable to that of AdMKE1(19kdel) (Fig. 7a). The prominent number of released virus observed in the Adwild infection possibly due to the efficacious virus production peculiar to native viruses (Fig.7b), especially the fact that the WT virus contains E3, which is known to enhance cytolytic effect.

Discussion

The potential utility of Ad as a therapeutic oncolytic agent has been considered at least since the middle of 20th century. The accumulation of knowledge about Ad in recent years has seen the re-emergence of this concept with the development of CRAds for cancer therapy. The key concept of the CRAd strategy is achievement of tumor cell killing via tumor-specific virus replication (5-7, 28), and control of E1A expression by using a tumor specific promoter (tsp) is a promising method to confer upon Ads tumor specific replication properties (29,30). Many tsps, including MK promoter, have been evaluated for this approach, and a virus containing the prostate specific antigen (PSA) promoter has already been employed in human clinical trial (31).

We have been evaluating the development of therapeutic viral agents for advanced NBs or other pediatric tumors. The treatment of advanced NBs is one of the most difficult problems in the pediatric surgical field. Fortunately, NBs, even the case of primary tumors, are susceptible to the Ad infection (32) and several authors have proposed Ad vector-based gene therapy for these conditions (14,17,33). We have extended the potential use of Ads in this disease now to include CRAds based on the MK promoter. Our previous study illustrated the potential utility of the CRAd approach for bone marrow purging in a model system which has relevance for the high dose chemotherapy/bone marrow transplant approach for advanced NB or Ewing's sarcoma patients (4). However, NB SK-N-SH cell killing effect induced by AdMKE1 infection was lower than that observed in Ewing's sarcomas or Wilms' tumors.

The Ad 19k protein encoded in E1B region is an Ad homologue of Bcl-2 protein, an anti-apoptotic agent (34,35). Hay *et al.* reported that the replicating Ad without E1B 19k units showed enhanced cell-killing efficacy compared with the CRAds having an intact E1 region (21,22). Thus, we have modified the MK-CRAd by deleting the E1B 19kD protein coding region to improve the oncolytic efficiency against SK-N-SH cells. We presented in this report that AdMKE1(19kdel) actually killed SK-N-SH cells more effectively than AdMKE1. Especially, cell-killing effect of AdMKE1(19kdel) was comparable to that of AdWT. On the other hand, the MeWo cells which we chose as an Ad-infectable MK negative control showed insignificant damage by the AdMKE1(19kdel) infection. These data indicate that E1B modification in the context of a tsp-controlled CRAd is a useful candidate approach to improve oncolytic efficacy whilst retaining specificity.

A multitude of factors contribute to the oncolytic efficacy of CRAds, including the initial infectivity of the vector for the target cells (36,37), efficiency of viral replication within the cell and efficiency of release of the viral progeny and dissemination throughout the tumor mass. We speculated that a higher level of the viral replication might be observed in the cells infected with E1B modified CRAds compared with AdMKE1, because we believed that the viral replication induced the oncolytic effect on SK-N-SH and the viral replication speed was correlated with oncolytic potency. In the case of AdCMVE1, this did indeed seem to be the case – much higher levels of viral DNA were seen at early time points compared to the other viruses. However, the viral replication speeds of E1B modified CRAds were same or less than AdMKE1 in SK-N-SH host cells. Since the E1B 19k protein serves as an anti-apoptotic agent (34), it may be that the Ad vectors defective

in E1B 19k expression show improved cell killing effect without augmentation of the viral replication. As the previous report, the improvement of cell killing effect was mainly due to the early cell burst and early virus release from the NB cells.

It was also noted that Adwild could replicate and induce cell death most efficiently in spite of the lower level of E1A expression in the cells infected with Adwild vs AdCMVE1. We believe this is most likely due to the fact that the wild-type Ad contains E3 whereas the other viruses do not. In this regard, the death protein encoded in E3 region has been reported as the important trigger for the late phase cell burst (38). In the absence of E3, we speculate that E1A expression could play a central role in the cell killing mechanism. The E1A proteins are known as not only key triggers of Ad replication but also strong apoptosis agents *per se* (39,40). Thus, because the E1B 19k protein ordinarily acts to inhibit apoptosis induced by E1A proteins (41), the enhanced cell killing effect seen with AdMKE1(19kdel) may result from the unopposed apoptotic activity of E1A proteins. Re-introduction of E3 protein function into the AdMKE1(19kdel) CRAd (within the size constraints of the genome) may still further improve the utility of this agent.

The clinical application of CRAds is well underway in human trials. The basic safety appears to have been established, but in many cases efficacy has been disappointing. Efforts to improve efficacy by combining CRAds with conventional therapies are now being evaluated. We show here that further improvements in CRAd efficacy can be achieved through 19k deletion – importantly without any loss of specificity of cell-killing

effect. Thus, this approach may be one of several improvements that together achieve greater therapeutic utility for cancer treatment, and in the current context for neuroblastoma (and other MK positive tumors) in particular.

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Figure Legends

Figure 1. Schema of the Ad vectors used in this study. All vectors shown here are constructed from E3 region (nucleotide 28130 to 30820 of Ad genome) deleted Ad type 5 backbone. Ad E1A region is essential to the virus replication, non-replicating virus (AdMKLuc) due to the replacement of Ad E1 region (nucleotide 324-3533 of Ad genome) to MK promoter-luciferase gene sequence. AdMKE1, AdCMVE1, and AdMKE1(19kdel) are the CRAd vectors used in this study and do not contain the Ad E1A promoter region expanding from nucleotide 324 to 488 of the Ad genome. AdCMVE1 and AdMKE1 differ in the promoter driving E1A expression. AdMKE1(19kdel) is defective in the E1B 19kD protein coding region. We also used an adenovirus type 5 as a wild type Ad control (Adwild).

Figure 2. Evaluation of Ad viral E1 expression in G-401 cells after Ad vector infection. M stands for a marker DNA. Lane1, AdMKLuc; Lane 2, AdMKE1; Lane 3, AdMKE1(19Kdel); Lane 4, AdCMVE1; Lane 5, Adwild. These data meant that each Ad vector expressed the anticipated E1 mRNAs.

Figure 3a. Evaluation of E1A expression in the SK-N-SH cells after Ad vector infection by virtue of RT-PCR method. M stands for a marker DNA. Lane1, AdMKLuc; Lane 2, adMKE1; Lane 3, AdMKE1(19kdel); Lane 4, AdCMVE1; Lane 5, Adwild; Lane 6, mock infection.

Figure 3b. Quantitative evaluation of E1A transcripts after Ad vector infection. the quantitative real-time PCR method was available for the measurement of the E1A mRNA.

Figure 4. Oncolytic efficacy induced by the infection with CRAds and controls. Cell killing efficiency was evaluated by the crystal violet staining. Survived cells on 8 days after Ad vector infection were stained in violet.

Figure 5. Oncolytic efficacy evaluated by MTA assay. The formazan product was measured by absorbance (OD) at 490 nm. We made blank controls of culture medium without cells and measured the absorbance (blank OD) after MTS procedure. ΔOD =OD – blank OD.

Figure 6. Assessment of viral DNA replication in course of time after infection. Since all vectors tested here contained complete E4 region, the viral DNA replication rate was evaluated by measuring the E4 copy number using the quantitative real-time PCR method. Data are means of triplicate determinations.

Figure 7. Encapsidated virus DNA copy number in SK-N-SH supernatants. Data are means of triplicate determinations. E4 copy number was measured as Ad virus DNA copy number. A), the results 3 days after infection. B), the results 5 day after infection.

APPENDIX F

Towards a new generation conditionally replicating adenoviruses: paring tumor selectivity with maximal oncolysis

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Running title:

Towards a new generation CRADs

Abstract

Conditionally replicating adenoviruses (CRADs) represent a promising new platform for the treatment of cancer. CRADs have been demonstrated to kill tumor cells where other therapies fail indicating that their antitumor properties are complementary to, and distinct from, standard treatments such as chemotherapy and radiation. In clinic trails CRADs have shown encouraging results, demonstrating mild side effects when administrated at high dosages and via different routes, including intratumorally, intraperitoneally and intravenously. Tumor-selective replication has been detected, although as a single agent the efficacy appears to be rather limited. Interestingly, combined treatment with radiation or chemotherapy has been found to enhance CRAD efficacy considerably. To date, the molecular mechanisms underlying adenovirus-mediated oncolysis, and the way in which chemotherapy enhances oncolysis is not well understood. A fuller knowledge of these processes will open up new strategies to improve the cell killing potential of CRADs. Here, we discuss several possibilities that may lead to CRADs with enhanced oncolytic activity. These approaches include strategies to functionally couple tumor targeting and optimal oncolytic activity, and ways to further increase tumor cell disruption at later stages of infection in order to facilitate the spreading of virus throughout the tumor mass. In addition, improved methods to evaluate the efficacy of these agents in animal models, and in the clinic will be required, in order to systematically test and optimise CRAD efficacy, also taking into account the influence of tumor characteristics and administration route.

Conditionally replicating adenoviruses

The concept of using replication competent adenoviruses for the treatment of cancer, also known as adenoviral therapy, originated in the 1950's. The knowledge that adenoviruses could eliminate cancer cells *in vitro*, as a consequence of their reproductive cycle leading to cell lysis ("oncolysis"), resulted in clinical studies in which various wild-type adenoviral serotypes were examined for their effect on cervical cancer patients (Smith *et al.*, 1956). In the studies, no significant toxicity was reported following intratumoral injection or intravenous administration and a moderate tumor response was observed. It wasn't until 1996 that this concept regained attention via the use of a genetically engineered adenovirus with tumor selective replication characteristics, leading to the first CRAD that was developed for cancer therapy (Bisshoff *et al.*, 1996). It is now broadly recognized that these agents have beneficial properties when compared to their non-replicating counterparts that were initially used for cancer gene therapy.

In this regard, cancer gene therapy with non-replicating adenoviruses, although promising in preclinical models, has not resulted in successful treatments in the clinic. Armed with various therapeutic genes, including prodrug-converting enzymes and tumor suppressor genes, these agents have not demonstrated the expected beneficial effects vis à vis the eradication of cancer cells in the human clinical context. The main reason for the poor clinical therapeutic effect of these agents is related to the relatively small number of cancer cells in the tumor mass that are transduced by these vectors *in vivo*. As a consequence, the levels of expression and the dispersion of the therapeutic genes limits the clinical effect.

The new therapeutic platforms provided by CRADs are expected to overcome these limitations by their ability to increase the input dose of the therapeutic gene, and moreover, by the oncolysis and eradication of cancer cells during replication. In clinical trails, CRADs have shown to be promising and safe agents. On the other hand, in clinical trails as single agents their antitumor effects have been somewhat disappointing. Thus, these clinical studies have been of great importance for defining the system's current limitations. In this review, we address these limitations and focus on advancing several strategies that may improve the tumor killing properties of CRADs resulting in the development of agents with enhanced therapeutic potential.

CRADs in human clinical trails

The most studied CRAD so far is the one originally generated in the lab of Frank McCormick (Bishoff *et al.*, 1996), designated *dl*1520, also known as ONYX-015, and recently renamed CI-1042 (Pfizer Corporation, Groton, CT, USA). In this CRAD the viral gene E1B-55kD has been deleted which is required for binding to, and inactivation of, the p53 protein that is essential for virus replication. Being part of a cellular antiviral mechanism, the p53 protein would normally trigger a cellular response leading to early death of the host cell thereby preventing replication and spreading of the virus. In cancer cells that often lack functional p53, E1B-55kD would be dispensable, thus resulting in selectivity, a concept that has been confirmed in preclinical studies (Heise *et al.*, 1997). However, on this basis reduced replication and cytopathogenicity has been reported for this CRAD when compared to wild-type virus (Bishoff *et al.*, 1996; Harada and Berk, 1999). The clinical tests of this oncolytic agent in various cancer types, including head and neck and pancreatic cancer, have been evaluated extensively in recent reviews in terms of safety and efficacy (Alemany *et al.*, 2000; Kirn, 2001). The overall conclusion is that adenoviral therapy is a safe method when applied via various routes. Further, tumor-selective replication has been documented, thus validating the concept *in vivo*. Of note, no evidence was obtained for the expected reduced efficacy in patients with pre-existing antibodies against adenoviruses (Ganly *et al.*, 2000; Nemunaitis *et al.*, 2000). In fact the immune response is generally considered to be a factor that can increase the antitumor effect of the therapy. However, CRADs as a single agent have demonstrated limited efficacy with an overall response rate of approximately 15 % in patients receiving the agent. Interestingly, the efficacy could be significantly enhanced by combined treatment with chemotherapy.

The results obtained in these studies have been very helpful in determining the limitations of the current generation of CRADs, and in determining which aspects need to be addressed in order to develop a new generation of improved agents. In this respect, critical problems that have been encountered are: i.) The infectivity of cancer cells by adenovirus; ii.) Tumor selectivity of CRAds in relation to efficacy; iii.) Oncolytic activity or cell-death-inducing ability of CRADs; iv.) Accessibility of tumor for virus internalization and spreading; v.) Methods to evaluate CRAD efficacy in animal

models and in the clinic. In the following we will elaborate in more detail on these aspects of CRAD efficacy.

Adenovirus infection of cancer cells

Apart from the favorable characteristics of CRADs compared to the use of non-replicating adenovirus vectors some problems are common to both approaches. A major issue is the fact that cells can be resistant for adenovirus infection due to lack of the primary receptor for viral entry, the coxsackie-adenovirus receptor (CAR) (Wickham *et al.*, 1996; Douglas *et al.*, 1996). It has been noted that primary tumor cells often express relative low levels of CAR resulting in poor infectivity, which in case of CRADs will also affect the lateral dispersion of the virus in tumor tissue. This has been demonstrated by analyses of the oncolytic activity of wild-type adenovirus in a pair of tumor cell lines that differed only in CAR expression levels, demonstrating that low CAR levels strongly reduced viral replication and oncolysis in monolayer cultures and murine tumor models (Douglas *et al.*, 2001). To circumvent this, CAR-independent entry pathways have been identified that can bypass this deficiency, such as the use of the RGD motif in the fiber knob of the virus that facilitates binding and entry via integrin receptors that are abundantly expressed on tumor cells (Dmitriev *et al.*, 1998; Krasnykh *et al.*, 2000). CRADs have been generated containing fiber knobs with intact CAR-entry and an additional integrin-entry capability resulting in more effective antitumor characteristics by enhanced infectivity (Suzuki *et al.*, 2001). Additional strategies have been explored for obtaining tumor specific entry of adenoviruses, involving the modification of the viral coat or the use of secondary targeting moieties, approaches that have been reviewed in more detail elsewhere (Curiel, 1999; Wickham, 2000).

Selectivity of CRADs

Exploitation of replicating adenoviruses as a new modality for cancer gene therapy has lead to the use of novel ways to obtain tumor selectivity. CRADs that are now available make use of the frequent inactivation of tumor suppressor genes in cancer that occur as part of the process leading to malignant transformation, the so-called type 1 CRADs (Curiel, 2000). Examples are mutations or deletions in the

p53 and Rb genes, their protein products known to interact with, and to be modulated by, adenoviral gene products as an essential step in virus propagation. However, the exploited strategies for obtaining selectivity are often at the expense of efficacy as a consequence of the adenoviruses reproductive cycle being a highly orchestrated process. At the molecular level, completion of the infectious cycle relies on the timely expression of a set of regulatory proteins that interact with essential endogenous cellular pathways that determine cell viability in order to facilitate viral DNA replication, expression of adenoviral genes and finally disruption of the cell and the release of new viral particles (Yeh and Perricaudet, 1997). Specifically, the viral genome encodes eight transcriptional units that are activated in a timely way at different phases of the infection, referred to as immediate early (E1A), early (E1B, E2, E3 and E4), intermediate (pIX and Iva2), and late genes encoding structural proteins for the capsid and the internal core. The early genes are mainly regulatory proteins that set the stage for viral DNA replication thereby blocking cellular antiviral strategies such as the activation of cell death programs and the down regulation of immune response stimulatory proteins, strategies that are shared by other mammalian DNA viruses (for review see Wold *et al.*, 1999). At all stages of infection adenovirus proteins control various cellular processes by interacting with multiple host cellular proteins, many interactions of which are not yet completely understood or remain to be identified.

Upon infection, the immediate expression of E1A, and its binding to Rb, leads to the release of the transcription factor E2F which forces the host cell to enter the S-phase of the cell cycle in order to facilitate the co-replication of the viral genome (Flint and Shenk, 1997). Infection, E1A expression, and the unscheduled entry of the cell into the S-phase inflict cellular stress signals leading to the activation of cell cycle checkpoints and the onset of suicide pathways or programmed cell death (PCD), also known as apoptosis. Of note, the p53 protein plays an important role in the activation of apoptosis in the infected cell. In opposition to these processes, the adenoviral proteins E1B-55kD and E4orf6 work in concert to bind to p53 causing its degradation, thereby facilitating host cell survival (Steegenga *et al.*, 1998). The exploitation of the interaction between E1B-55kD and cellular p53 for obtaining tumor selectivity with *dl*1520 has caused considerable controversy in the field. It has been reported that the wild-type virus grows more efficiently in cells expressing wild-type p53 when compared to *dl*1520 in p53-mutated cells, suggesting that either functional p53 is required for effective

replication and/or that E1B-55kD-p53 interaction has a favorable effect (Hall *et al.*, 1998; Ridgway *et al.*, 1997; Dix *et al.*, 2000). In addition, several groups have demonstrated that the host range specificity of *dl*1520 in *in vitro* models is independent of p53 status (Rothmann *et al.*, 1998; Goodrum and Ornelles, 1998; Turnell *et al.*, 1999; Harada and Berk, 1999). One reason for these discrepancies may be that in most studies cells derived from various cancer types were compared for examining the relationship p53 status and *dl*1520 replication rather than using isogenic cell systems to exclude the involvement of other genetic factors. In this regard, Rogulski and co-workers (Rogulski *et al.*, 2000) showed that in an isogenic colorectal cancer cell model *in vivo dl*1520 replication occurred in both p53 wild-type and mutant cells, though with significant higher antitumor activity in p53 deficient tumors. Another cause for these differences is due to the multifunctional properties of p53 and the particular function being inactivated in the cancer cell studied. In a recent study in hepatocellular carcinoma cell lines, it was found that an intact transcription activator function of p53 in mutants leads to increased susceptibility for *dl*1520 when compared to p53 mutants in which this function was disrupted (Zhao *et al.*, 2001). Also other factors in the p53 pathway have been identified that affect the oncolytic properties of *dl*1520, such as deletions in the INK4a/ARF locus, which occur at high frequency in cancer cells (McCormick, 2000). This locus encodes two proteins, $p14^{ARF}$ and $p16^{INK4a}$, that are part of the pRb and p53 pathways, respectively. $P14^{ARF}$ can be activated by E2F that, in its turn promotes, the degradation of MDM-2, a cellular protein that prevents p53 accumulation in a manner similar to E1B-55kD, leading to activation of p53. In this way, $p14^{ARF}$ links the pRb with the p53 pathway and thus connects adenovirus-dependent activation of the pRb route with the p53 response. Recently it was shown that a functional $p14^{ARF}$-p53 pathway is required for inhibiting ONYX-015 replication and that disrupted $p14^{ARF}$ function facilitates replication (Ries *et al.*, 2000). Also in mesothelioma cells with wild-type p53, mutated $p14^{ARF}$ enabled ONYX-015 replication whereas restored expression of functional $p14^{ARF}$ significantly increased resistance to oncolyisis (Yang *et al.*, 2001). These studies illustrate the relative lack of knowledge on the cellular mechanisms underlying tumor-selectivity of type 1 CRADs, such as ONYX-015, and the ongoing identification of relevant cellular factors. Another promising CRAD, not yet tested in the clinic, is the Ad-delta24 that makes use of the function of the E1A protein to bind to Rb in order to trigger cell cycle progression into the S-phase (Fueyo *et*

al., 2000). Independently, a similar CRAd has been developed, designated *dl*922-947 (Heise *et al.*, 2000a). These CRADs are designed for selective replication in tumor cells that have a deficiency in the Rb pathway, which is the case in the majority of cancers, by deleting 8 amino acids in conserved-region 2 (CR2) of E1A that are required for binding to pRb and related pocket proteins (Dyson *et al.*, 1992). In addition, an infectivity-enhanced variant containing the RGD targeting motif in the fiber knob of the virus has been generated with even more favorable antitumor characteristics in lung and prostate cancer cells (Suzuki *et al.*, 2001). Both CRADs are very effective in eradicating various types of cancer cells in preclinical studies, in most cases more effectively than *dl*1520 or wild-type virus (Heise *at al.*, 2000a). However, a recent study employing an organotypic model derived from human primary keratinocytes for examining the effect of various E1A mutants for replication and their potential to kill cells, indicated that Ad-delta24 is not as selective as anticipated (Balague *et al.*, 2001). While studying the complementary activity of HPVE6 and E7 proteins towards the E1A deletion mutants, normal and E6E7 expressing keratinocytes facilitated the replication of Ad-delta24 as efficiently as wild-type adenovirus. In addition, a CRAD carrying an additional deletion in CR1, a region also known to be involved in binding to members of the Rb pocket protein family, demonstrated substantial selectivity for HPV protein-expressing cells with a strongly reduced but not completely abrogated replication in normal cells.

The superior antitumor activity of Ad-delta24 is likely to be due to the fact that only a small, but specific, mutation in E1A was introduced without altering other functions of E1A. In a similar way, an improved version of *dl*1520 may be generated by making more precise mutations in the E1B gene. Apart from binding to p53 and E4ORF6, E1B-55kD is known to facilitate the transport of late viral mRNAs from the nucleus to the ribosomes and a nuclear export signal has been identified in this protein controlling nuclear/cytoplasmic export (Kratzer *et al.*, 2000). Impaired mRNA transport in the E1B-55kD-deleted CRAD *dl*1520 is probably the cause for its reduced potency when compared to wild-type adenovirus or Ad-delta24 (Harada and Berk, 1999). A recent report describes the identification of an E1B-55kD mutant R240A that fails to degrade p53 but has retained its E4orf6-binding and mRNA transporting potential (Shen *et al.*, 2001). According to the expectation, this

mutant in the context of a replicating adenovirus was demonstrated to enhance replication and to be effective in a broader range of cell types when compared to *dl*1520.

The above studies on selectivity and efficacy of CRADs indicate that it is possible to generate CRADs with improved specificity and activity by introducing more precise mutations in adenoviral genes that only affect the critical and desired functions facilitating tumor preferentiality. In order to derive such CRADs, a better knowledge of the function of the viral genes will be required. On the other hand, it is also evident that the development of such CRADs may actually be at the expense of selectivity of these agents thereby compromising safety issues for applications in the clinic. Additional safety measures will need to be incorporated if true selectivity cannot be obtained in this way, for example, by making use of tumor/tissue specific promoters (TSPs). Indeed, apart from CRADs that make use of mutations in adenoviral genes that can be rescued in cancer cells, CRADs have been generated in which essential adenoviral genes are driven by TSPs, also known as type 2 CRADs (Curiel, 2000). The use of TSPs that direct the expression of genes essential for viral replication leading to the transcriptional targeting of viruses to cancer cells have been widely exploited (for review see Gomez-Navarro *et al.*, 2000; Kirn *et al.*, 2001a). For driving E1A expression, multiple promoters have been used for specific cancers such as the prostate-specific antigen (PSA) promoter for prostate cancer and the alpha-Fetoprotein (AFP) promoter for hepatocarcinomas (Rodriquez *et al.*, 1997; Alemany *et al.*, 1999). An additional favorable feature of using TSPs is to prevent potential replication at unwanted sites in the body, such as in the liver in which adenoviruses accumulate via specific and non-specific interactions. There is an ongoing quest for promoters that display "tumor-on" and "liver off" features. Several have been employed for mediating tumor specific expression of suicide genes, including the cyclooxygenase-2 (Cox-2), Midkine (Mk) and telomerase reverse transcriptase (hTert) promoters, which may also be suitable for use in the context of a CRAD (Yamamoto *et al.*, 2001; Aduchi *et al.*, 2000; Majunder *et al.*, 2001).

CRAD-induced cell death

Intensive research during the last decade has revealed that PCD is a genetically controlled process with many cellular factors involved in sensing and balancing survival and death-inducing stimuli. This

balance can be disturbed in various ways, including by cytotoxic agents, radiation, growth factor withdrawal and virus infection. The molecular events triggering PCD, as well as the accompanying cellular characteristics, can vary greatly between different cell systems and in relation to the type of stimulus, classical apoptosis being one of them.

Several adenovirus encoded gene products have been found to either block or activate cell death, and the coordinated and timely expression of these factors lead to optimal conditions for generating progeny viruses. Amongst the inducers of cell death are the E1A proteins, the E4 region encoded proteins ORF4 and ORF6/7 and the E3-11,6kD protein, also known as the adenovirus death protein (ADP), whereas E1B-19kD, E1B, E4ORF6 suppress cell death (for recent review see Braithwaite and Russel, 2001). Except from ADP, the gene products encoded by the E3 region act to prevent cell death induced by external stimuli such as factors from the immune system (for review see Wold *et al.*, 1999). ADP appears to be the only adenovirus-encoded protein that directly affects cell lyses, whereas the other gene products modulate cell survival through the existing pathways present in the host cell. The latter include the p53 pathway and the Bcl2-family of pro- and anti-apoptotic proteins that control mitochondria integrity, a crucial factor in the regulation of PCD (Kroemer, 1997; Green and Reed, 1998). In this regard, a window of opportunity for enhancing the antitumor potential of CRADs is at the final stage of the reproductive cycle that involves the lysis of the host cell and spreading of viral progeny. Optimal oncolysis in cancer cells may mandate other, or additional, stimuli than required for optimal reproduction in natural host cells that are located in the upper and lower respiratory tract in case of adenoviruses belonging to subgroups B and C. One may argue that adenovirus reproduction in cancer cells, that are often resistant at the start of therapeutic treatment, or have acquired resistance to multiple forms of anticancer agents during treatment, will also be in one or more ways refractory to adenovirus-induced cell death. Thus far, only the enhancing effect of ADP on adenovirus-induced oncolysis has been established in this regard; ADP is transcribed at low levels at early stages from the E3 promoter, whereas at later stages the major late promoter facilitates high expression levels of ADP (Tollefson *et al.*, 1992). The mode of action of ADP, an integral membrane glycoprotein, is not well understood (Scaria *et al.* 1992), however, adenoviruses lacking functional ADP have been shown to kill cells and release progeny virus more slowly than wild-type adenovirus (Tollefson *et al.*, 1996).

Doronin and co-workers (2000) have shown that overexpression of ADP, in the context of a CRAD with small-deletions in the E1A region, is more potent in eradicating tumor cells than versions lacking ADP.

The notion that altering the cell death-inducing properties of adenoviruses may lead to improved oncolytic potential has also been tested by deleting adenovirus genes that have an antiapoptotic function, such as the Bcl-2 homologue E1B-19kD. Realizing that cancer cells often have developed cell death inhibitory mechanisms during malignant transformation, Sauthoff and co-workers (2000) have generated a viral mutant in which E1B-19kD has been deleted which was shown to be more effective at tumor killing than wild-type virus. This variant displayed a more rapid release of viral particles from infected tumor cells in monolayer compared with the wild-type virus, likely due to enhanced PCD, and was more potent in a lung cancer xenograft model in mice (Harrison *et al.*, 2001).

Another strategy involves the use of cytotoxic genes or pro-apoptotic genes in the context of a CRAD. Combining the prodrug-enzyme suicide gene strategy, ganciclovir (GCV)/ thymidine kinase (TK), with an E1B-55kD-deleted virus was found to be more effective in killing tumor cells than control virus without TK (Wildner *et al.*, 1999). Interestingly, GCV treatment failed to enhance the efficacy of a replicating adenovirus expressing functional E1B-55kD in combination with TK, whereas E1B-55kD deleted viruses showed an increased cytophatic effect following GCV treatment, perhaps due to already optimal conditions and enhanced baseline of oncolytic activity in the previous agent (Wildner and Morris, 2000). In addition, the incorporation of the pro-apoptotic tumor necrosis factor (TNF) gene driven by the CMV promoter in a replicating adenovirus, rendered breast cancer specific by employing the MUC1 promoter, showed enhanced oncolytic activity compared to the TNF-deleted version (Kurihara *et al.*, 2000).

Although these approaches in the models described above appeared to be of utility it is realized that the optimal effect confered by an incorporated cell death-inducing gene may depend on the timing of its onset of expression. Effective replication of the adenovirus is dependent on the coordinated and timely expression of adenoviral genes; inappropriate timing of expression of a death-inducing gene may be counterproductive to the cycles of infection. This is illustrated by the complex interactions observed between the oncolytic effect of a replicating vaccinia virus and the cytosine deaminase (CD)

/5-FC enzyme/produg system (McCart *et al.*, 2000). The investigators found that the tumor response enhancing effect of 5-FC was virus dose dependent; at low multiplicity of infection (MOI) the prodrug enhanced the response whereas at higher MOI (> 0.1) a decrease in efficacy was evident.

A likely hypothesis is that the induction of expression of cytotoxic genes in a replicating virus would be most effective at later stages of infection in order to enhance the outburst of viruses in cell death resistant cancer cells. Recent papers from Hermiston and colleques have dealt with this issue by placing toxin genes in the E3 region in place of the native adenoviral genes, in consideration of the fact that the majority of genes in this region are not essential for viral replication *in vitro*. They found that the replacement of toxin genes for the E3-6.7/gp19K, ADP and E3B encoding regions resulted in similar timing of expression as the replaced viral genes thereby maintaining normal expression of the resident adenoviral genes (Hawkins *et al.*, 2001; Hawkins and Hermiston, 2001; Hawkins and Hermiston, 2001a). Although the various viruses containing as replacements a cDNA encoding CD or TNFalpha were not evaluated in detail for their oncolytic activity, the expression of these genes was stronger than obtained with the CMV promoter, probably due to the high copy numbers of viral DNA at later stages. Moreover, deletion of ADP resulted in a longer survival of the infected cells accompanied with attenuated protein synthesis leading to an increased production from the inserted gene (Hawkins and Hermiston, 2001). These studies appear to indicate that the E3 region is an attractive locus for inserting potent death-inducing genes in the CRAD genome, thereby not interfering with viral replication but facilitating the disruption of possibly death-resistant cancer cells to obtain optimal dispersion.

CRADs and chemotherapy

Several investigators in various *in vitro* and *in vivo* model systems, as well as in the clinic, have reported the additive or synergistic effects between chemotherapy and CRADs. In a phase II trail for patients with recurrent head and neck cancer, *dl*1520 (ONYX-015), in combination with cisplatin and 5-fluorouracil, had the strongest antitumor effect when compared to the separate treatments (Khuri *et al.*, 2000). Studies to characterize this interaction in nude mice-human tumor xenografts models indicated that this synergism was independent of the route of administration and p53 status. However,

the order of administration of the agents appeared to be crucial; treatment with ONYX-015 first, or the simultaneously exposure to cisplatin and the virus being superior to cisplatin followed by ONYX-015 (Heise *et al.*, 2000). Contrary to the lack of involvement of p53, You *et al.* (2000) found in monolayer lung cancer cultures cells with non-functional p53 to be at least 10 times more sensitive to ONYX-015 cytolysis than cells with wt p53, and chemotherapy with taxol and cisplatin only being able to enhance oncolysis in p53 mutant lung cancer cells. Synergistic effects have also been observed in prostate cancer cells, both *in vitro* and *in vivo*, upon treatment with CV787, a prostate cancer-specific replicating adenovirus and the taxanes paclitaxel and docetaxel (Yu *et al.*, 2001). In addition, radiotherapy has also been found to enhance the antitumor activity of ONYX-015 in xenograft mice models (Rogulski *et al.*, 2000). Using isogenic cell lines with wt or mutant p53, the authors observed no effect of irradiation on viral DNA replication in monolayer cultures. However, *in vivo* little oncolytic activity was found in p53 wt tumors, with radiation having no enhancing effect, whereas in mutant p53 tumors the already higher antitumor basal level was further increased following irradiation.

Classically, synergy is defined as greater than additive therapeutic effects when compared with the therapeutic efficacy of each drug alone. Drugs with dissimilar mechanisms of action that target two independent pathways will likely result in enhanced toxicity, either additive or synergistic. The molecular mechanism underlying the synergy between replicating adenoviruses and chemotherapy are currently unknown. For the *in vivo* tumor context, this interaction may involve the enhanced activity of the immune system due to chemotherapy-dependent tumor cell damage or may perhaps be due to anti-angiogenic effects elicited by these cytotoxic agents. In addition, it could be envisioned that chemotherapy may alter the structure of the tumor mass thereby facilitating the penetration and spreading of virus throughout the tumor. However, the interaction between the two types of anticancer agents can also occur at the cellular level in the absence of an immune response and a structural context, as for example illustrated by the synergy found between ONYX-015 and subtoxic concentrations of paclitaxel or cisplatin in lung cancer cell lines and primary lung cancer cells (You *et al.*, 2000) and CV787 in prostate cancer cells (Yu *et al.*, 2001).

The molecular mechanisms underlying the enhancing effects of chemotherapy and irradiation on CRAD efficacy are not well understood, however, the possibility exists that viral genes interact with host cellular factors thus facilitating the tumor cell killing effect of CRADs. For example, the two E1A variants consisting of 289 (289R) and 243 (243R) amino acids proteins can induce apoptosis via both p53-dependent and independent mechanisms (White, 1998); 243R-induced apoptosis requires the presence of p53 leading to deregulation of Bcl-2 and Bax and subsequent apoptosis. The 289R product can trigger apoptosis independent from p53, however more recently also 243R has been reported to induce apoptosis independent of p53 (Putzer *et al.*, 2000). E1A expression has also been found to enhance the sensitivity to apoptosis induced by ionizing radiation and various cytotoxic agents in murine embryonic fibroblasts, keratinocytes and human ovarian cancer and leukemia cells (Stiewe *et al.*, 2000 and references therein). CRAD-mediated E1A expression may thus contribute to synergistic effects with chemotherapy or radiation, although also other adenovirus genes may be involved, which currently remains to be investigated. Results from such studies may be translated into the generation of CRADs with enhanced activity.

On the other hand, the basis of cancer cells resistance to chemotherapy-induced cell death, and the consequent effect on the oncolytic potential of CRADs, has currently not been addressed. Interestingly, in a recent study with cisplatin-sensitive and -resistant p53 mutant expressing ovarian cancer cell lines, ONYX-015 displayed preferential replication in cisplatin-resistant cells in *in vitro* and *in vivo* models (Ganly *et al.*, 2001). The restored expression of wild-type p53 in the sensitive cell line resulted in early onset of apoptosis that probably formed the basis for the observed reduction of viral production. These findings provide more evidence for the concept that early onset of cell death is detrimental for virus production whereas cell death resistance may delay or block viral release.

Tumor characteristics and other barriers for CRAD efficacy

In the ideal situation, a CRAD should be applied intravenously and, upon reaching the tumor site(s), infect tumor cells and spread throughout the tumor to eradicate all cancer cells, even when dealing with advanced, metastatic disease. Although these features have been very challenging to meet for all anticancer therapies, CRADs encounter several barriers that are particular for this class of agents. In

this respect, adenoviruses applied systemically are cleared for 90% within 24 hr by the liver as determined in both immune-competent and –deficient mice (Worgall *et al.*, 1997). In addition, mainly due to clearance by Kupffer cells in the liver, adenovirus half-life is approximately 2 minutes (Worgall *et al.*, 1997; Alemany *et al.*, 2000). In this context, the immune system may play a dual role, on one hand interfering with the virus function via neutralizing antibodies or by macrophage-mediated phagocytosis. On the other the virus may induce tumor-specific CTL responses that help lysing tumor cells as distinct from the oncolytic affect of the virus. Hence, the interactions between the growing tumor, the amount of replicating viruses and antiviral immune responses are highly complex and non-linear; attempts have been made to make predictions in a mathematical model (Wodarz 2001). In this model, the outcome of viral therapy is dependent on the balance between several host and viral parameters, including the growth and death rate of infected and non-infected tumor cells and the speed of viral replication. Such a model predicts an optimal antitumor activity at the highest possible level of oncolytic activity and in the absence of an immuneresponse. Also a fast growth rate of the tumor is expected to reduce efficacy and conventional treatments such as chemotherapy may be combined to decrease tumor growth and allowing the virus to eliminate as much tumor cells possible (Wodarz, 2001).

Other characteristics of the tumor mass may also obstruct the spreading of the CRAD such as the architecture of the tumor. In general it has been noted that established tumors appear to be more difficult to eliminate by CRADs than when tumor cells are pre-mixed with virus prior to injection in mice for xenograft models or when treatment is started early after tumor growth is detected. "Older" tumors are more difficult to eradicate due to infiltrating macrophages and fibroblasts resulting in connective tissue formation, the in-growth of blood-capillaries, the tumor matrix, and the presence of necrotic areas, that all impose blocks for the spreading of CRADs. It is currently unknown to what extent these obstacles interfere with CRAD efficacy but they will need to be addressed.

Validation of CRADs in animal models and in the clinic

The validation of CRAD infection, replication and selectivity are important measures to evaluate the efficacy of the agent. A mayor limitation for assessing the efficacy of CRADs in animal models is the

inability of CRADs derived from human serotypes to replicate in non-human tissue. CRAD activity can thus only be determined in human xenografts models, predominantly in mice, in terms of antitumor effect and general toxicity of the virus. On this basis, determination of CRAD selectivity remains unaddressed. The molecular basis of the inability for human CRADs to replicate in non-human host cells is currently unknow. Until this is resolved and animal cells can be modified to become replication permissive, an alternative may be the generation and use of species-specific CRADs, such as a murine adenovirus in mice. In the clinic, viral infection has been determined in tumor biopsy samples by *in situ* hybridization with adenoviral DNA. Replication was demonstrated by testing blood from patients using quantitative PCR for the presence of adenovirus sequences at day 3 after treatment, thereby assuming that this reflects replicating virus since the initial inoculate at this time will be cleared from the blood. Determining infection and replication in adenovirus in tumor biopsies by histologic analysis has its drawbacks. Biopsies only provide a small amount of tissue, thus increasing the chance for false negatives not to mention the ethical and practical matters associated with acquiring samples. For these reasons PCR is the method of choice, although both methods can be complementary.

The use of non-invasive methods to determine infection and replication is an important area of research that may help to evaluate CRAD efficacy. Methods that are currently developed are adenovirus imaging systems for *in vivo* detection of infected cells such as by incorporation of a transgene expressing the receptor for somatostatin subtype-2 (SSTR2) into the virus allowing detection with intravenously administered radiolabeled tracer (Rogers et al., 1999). The HSV-TK cassette has been used in combination with this approach also allowing the detection of TK upon exposure of cells to ^{131}I-FIAU with γ-camera imaging (Zinn *et al.*, 2001). Other strategies employ photon-emitting reporter genes, including luciferase and green fluorescent protein in conjunction with a charged-coupled device (CCD) camera for in vivo imaging (Honigman *et al.*, 2001 and references therein).

Future directions for improvements

The current limitations of CRADs for being effective single anticancer agents occur at multiple levels, including macro-cellular, cellular and molecular. Effective systemic delivery of these agents, being preferable in more advanced metastatic cancer, is hampered by clearance of the virus and binding/ uptake by non-tumor cells. The development of strategies to un-target the liver and other organs, in order to obtain best as possible tumor/ non-tumor targeting ratios are crucial for this application. This will require ongoing efforts to modify the viral coat to detarget the liver at the same time increasing tumor specificity via tumor-targeting approaches such as the incorporation of targeting domains in the fiberknob or the use of targeting moieties, including bispecific single chain antibodies. At the same time these methods will bypass the often-occurring CAR-deficiency of cancer cells. These approaches may be used in conjunction with TSPs that can drive both essential adenoviral genes and/or therapeutic genes to obtain liver off/ tumor on characteristics. Enhancing replication and oncolytic properties of CRADs to overcome possible structural barriers in the tumor will be necessary to improve tumor-eradicating activity. The optimization between selectivity and oncolytic activity by means of small and subtle gene deletion/ modifications, coupled with the use of TSPs, will probably provide the best and safest adenoviral platform to build in additional improvements. The combined use of enzyme/prodrug strategies or proapoptotic genes with such CRADs, thereby ensuring onset of expression at later stages of replication may improve oncolysis and spreading of the virus in the tumor mass and may help to overcome structural barriers. Moreover, for additional rationalized approaches to improve CRADs more basic research on the factors involved in adenoviral oncolysis will be required, including the unravelling of the mechanism(s) responsible for synergy between chemotherapy and CRADs that may be instrumental for designing better agents. Studies to understand the way in which rodent cells block adenovirus replication may lead to the development of transgenic mice models that are permissive for CRAD replication thus providing better model systems. Alternatively, other species- specific adenoviruses may used to examine CRAD characteristics. Finally, the incorporation of reporter genes in CRADs to allow non-invasive imaging techniques will be very helpful in determining CRAD efficacy.

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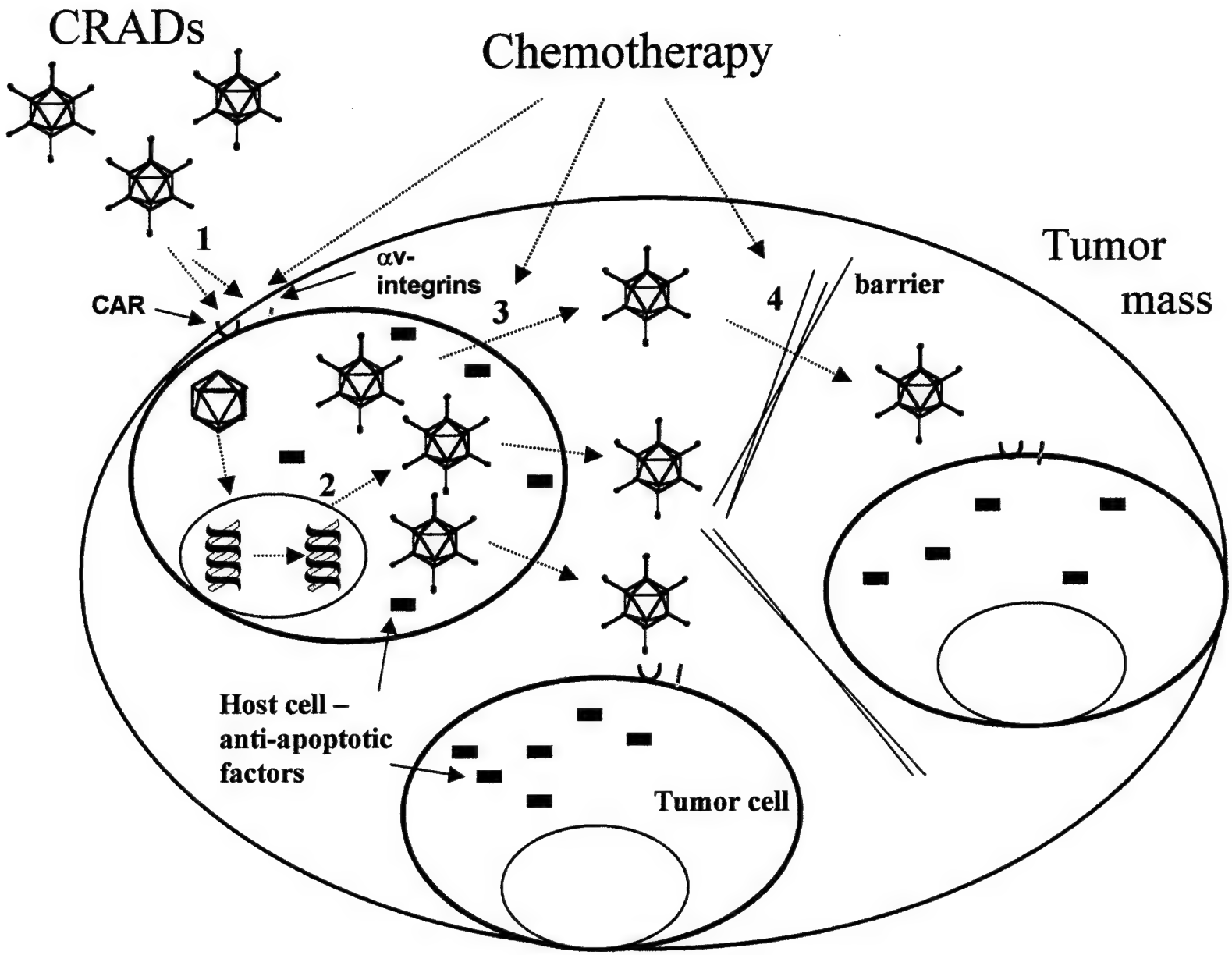
Legend to the Figure

Figure 1. Schematic representation of several conceptual obstacles for CRAD efficacy and the possible stages at which combined treatment with chemotherapy may sort its beneficial effect. Upon systemic administration of CRADs a fist hurdle may be difficulties to pass the extra cellular matrix of the tumor or the lack of adenoviral receptors on tumor cells (1). Combined treatment with chemotherapy may loosen the tumor structure thus facilitating CRAD penetration. The potential absence of CAR may be circumvented by exploiting other virus entry routes, including allowing primary entry via the αV-integrin-pathway by incorporation of the RGD motif in the fiber knob of the virus. After infection, both viral and cellular factors may influence the replication of the CRAD, such as gene-deletions in the CRAD genome in order to obtain tumor selectivity and/or host cell factors like p53 and additional yet to be identified factors (2). After viral assembly, cellular mechanisms conferring cell death or apoptosis resistance that are common to cancer cells may counteract cell death-induced by the CRAD and hamper the release of virus (3). At this stage chemotherapy may work in concert with the CRAD to enhance tumor cell killing, resulting in improved dispersion of the virus, however, may also act to disrupt possible existing intratumoral barriers (4). Alternatively, suicide or pro-apoptotic genes genetically-inserted in the CRAD and expressed at later stages of infection may stimulate tumor cell disruption and help to overcome possible barriers, thus leading to improved CRAD dispersion throughout the tumor. (See text for more details).


CRADs
Chemotherapy
1
αv-
integrins
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barrier
Tumor
mass
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Host cell –
anti-apoptotic
factors
Tumor cell

The flt-1 promoter for transcriptional targeting of teratocarcinoma[1]

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Key Word: cancer, gene therapy, adenovirus, transcriptional targeting, flt-1 promoter, teratocarcinoma

Running Title: Transcriptional targeting with the flt-1 promoter

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ABSTRACT

Flt-1, a receptor for vascular endothelial growth factor (VEGF), is known to display dysregulated expression in both tumor vasculature and tumor cells *per se*, suggesting that the flt-1 promoter might be a useful candidate to achieve tumor-specific

transgene expression. In addition, adenoviral (Ad) vectors containing transgenes under the control of the flt-1 promoter achieve very levels of expression in the normal liver, the major organ responsible for blood clearance of Ad and inadvertent transgene-related toxicity. Thus, we assessed the ability of Ad vectors containing the flt-1 promoter to achieve transgene expression in a range of gynecologic and breast tumor lines. High transgene expression levels were detected in teratocarcinoma lines, correlating with levels of flt-1 mRNA. These results suggest that the flt-1 promoter could be useful for transcriptionally targeted gene expression to teratocarcinoma, and that evaluation in other flt-1 positive tumors is warranted.

INTRODUCTION

A variety of cancer gene therapy approaches have been undertaken based on *in situ* molecular chemotherapy, where systemically administered prodrugs are locally converted to their toxic counterparts. Critical to the achievement of an acceptable therapeutic index is the restriction of toxin gene expression to tumor cells (1, 2). In this regard, ectopic vector localization, with consequent expression of the delivered genes at non-tumor sites, can induce treatment-limiting toxicities (3-5). These considerations are especially relevant for adenoviral (Ad) vectors, which exhibit a marked tropism for the liver when administered intravenously (3, 5). Thus, strategies to target transgene expression have been explored for Ad vector-based cancer gene therapy approaches (1, 4, 6). This strategy of transcriptional targeting is based upon the use of promoters that display preferential activity in tumor cells (7). Ideally, these promoters should be capable of substantially limiting the expression of transgenes in the liver as a means to mitigate

the potential toxicity of Ad-delivered toxin genes at this site. Thus, an ideal promoter for transcriptional targeting applications exhibits a "tumor on / liver off" phenotype when incorporated into an Ad vector (1). Tissue and tumor selective promoters have been defined which exhibit this desirable phenotype. In this respect, the gene for the vascular endothelial growth factor receptor type I (flt-1) has recently been shown to have dysregulated expression in tumors (8-10). Further, our recent studies have demonstrated that the flt-1 promoter exhibits a "liver off" phenotype when used in Ad vectors (11). These two considerations have suggested its utility as a promoter for use in Ad-based cancer gene therapy applications, including for gynecologic malignancies. Our studies imply that the flt-1 promoter is active in a subset of this class, specifically teratocarcinomas, suggesting that this promoter may be useful for gene therapy of a defined subset of cancers based on a common pathobiology.

MATERIALS AND METHODS

Cell culture. Hey, SKOV3.ip1 and OV-4 ovarian adenocarcinoma cell lines were kind gifts from Dr. Judy Wolf, Dr. Janet Price (both M.D. Anderson Cancer Center, Houston, TX) and Dr. Timothy J. Eberlein (Harvard Medical School, Boston, MA), respectively. The other cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). All cell lines were cultured in the recommended growth media and maintained in a humidified 37°C atmosphere containing 5% CO_2.

Viruses. Ad5flt-1luc1 and AdCMVluc1 are replication defective adenoviruses with a luciferase reporter gene, driven by the flt-1 or CMV promoters, respectively, in the E1 region (12). The viruses are isogenic and were propagated on 293 cells. Purification

was done with double CsCl gradients using standard methods and titered for viral particles (VP) with spectrophotometry. Functional titer (plaque forming units, PFU) was determined with plaque assay with an initial overnight infection of 293 cells. The viruses had the following titers: Adflt-1luc1 6.0 x 10^{11} VP/ml, 1.2 x 10^{10} PFU/ml, 50 VP/PFU; AdCMVluc1 9.4 x 10^{11} VP/ml, 1.9 x 10^{10} PFU/ml, 49 VP/PFU.

For the replication defective viruses Ad5flt-1LacZ and Ad5CMVLacZ the reporter gene is LacZ, driven by the identical promoters as described before (11). The viruses are isogenic and had the following titers: Ad5flt-1LacZ 2.0 x 10^{12} VP/ml, 2.0 x 10^{10} PFU/ml, 100 VP/PFU; Ad5CMVLacZ 5.0 x 10^{12} VP/ml, 5.0 x 10^{10} PFU/ml, 100 VP/PFU.

Luciferase assay. Cell lines were plated on day 1 at 25,000 cells per well on 24-well plates in 1 ml growth media (GM). On day 2, cells were infected with 5, 50 or 500 PFU/cell for 2 h in 200 µl 2% GM on a rocker. Afterwards, cells were washed once with 1 ml PBS and 1 ml GM was added per well. After 24 hours the GM was removed, cells were lysed with 200 µl lysis buffer (Reporter Lysis Buffer, Promega, Madison, WI) and freeze-thawed once. 20 µl of these samples was mixed with 100 µl of luciferase assay reagent (Reporter Lysis Buffer, Promega, Madison, WI) and measured with Berthold Lumat LB9501. Standardization was accomplished by setting the values obtained with CMV promoter as 100% for each cell line.

LacZ-staining. Cell lines were plated on day 1 at 50,000 cells per well on 24-well plates in 1 ml GM. On day 2, cells were infected with 500 PFU/cell for 2 h in 200 µl 2% GM on a rocker. Afterwards, cells were washed once with 1 ml PBS and 1 ml GM was added per well. After 24 hours, the GM was removed and cells were washed twice with PBS. Cells were fixed for 15 min with 0.5% glutaraldehyde and washed again twice with

PBS. Cells were stained for 2 1/2 hours with standard X-gal solution (containing 40 µl 2% X-gal, 10 µl 0.3 M potassiumferricyanide, 10 µl 0.3 M potassiumferrocyanide and 940 µl PBS per ml), washed again for 10 min with PBS and fixed a second time with 10% buffered formaline for 30 min. Pictures were taken by bright field microscopy at 10x magnification.

RT-PCR RNA of cells was extracted with RNeasy mini prep kit (Qiagen, Valencia, CA), treated with Deoxyribonuclease I (Life Technologies, Rockville, MD) for 30 min and RT-PCR of 90 ng RNA each was performed with the OneStep RT-PCR Kit (Qiagen, Valencia, CA) using the following primers: Flt-1 sense: 5'-TGC TTG AAA CCG TAG CTG G-3', Flt-1 antisense: 5'-GGT GCC AGA ACC ACT TGA TT-3'; GAPDH sense: 5'-TCC CAT CAC CAT CTT CCA-3'; GAPDH antisense: 5'-CAT CAC GCC ACA GTT TCC-3'. Preliminary serial dilution assays determined the linear range of amplification for the genes under investigation.

RESULTS AND DISCUSSION

In 1996 Abu-Jawdeh et al. (13) reported that the mRNA of the flt-1 receptor is strongly expressed in ovarian borderline and malignant neoplasms, although the cell type expressing the receptors was not further characterized. Studies in other tumors have identified both flt-1 and VEGF expression in malignant cells, suggesting the presence of an autocrine stimulatory loop promoting tumor cell growth in addition to the more commonly recognized effects of VEGF on tumor angiogenesis. Therefore, we decided to explore the flt-1 promoter as a potential candidate for transcriptional targeting of adenoviral gene therapy for breast or gynecologic cancer (14). Utilizing luciferase as a

reporter gene in a replication incompetent adenovirus, three ovarian adenocarcinoma cell lines (Hey, OV-4 and SKOV3.ip1), one ovarian teratocarcinoma cell line (PA-1), three breast cancer cell lines (AU565, GI-101A and ZR-75-1) and two cervical cancer cell lines (Caski and Hela) were infected with Adflt-1luc1 and the control vector AdCMVluc1, respectively, with 5, 50 and 500 PFU/cell. Only the teratocarcinoma cell line PA-1 exhibited significant flt-1 promoter activity when compared to CMV (14%; Figure 1).

We then expanded the analysis to include all commercially available teratocarcinoma cell lines [human ovarian (PA-1), murine ovarian (F9), and human testicular teratocarcinoma (NCCIT/NTERA-2)], and demonstrated activity of the flt-1 promoter in all of these lines (Figure 2). The relative values compared to CMV were 10% for NCCIT, 15% for PA-1, 33.5% for F9 and nearly 50% for NTERA-2. Thus, the flt-1 promoter appears to be active in teratocarcinoma, irrespective of whether the cell are of ovarian or testicular origin. Of note, NTERA-2 and F9 exhibited higher relative ratios than the flt-1 positive control cell line HUVEC (human umbilical vein endothelial cells) with 32%. Infection with 50 and 500 PFU/cell produced similar results (data not shown).

Theoretically, luciferase activity correlates more with promoter activity than the number of infected cells. Thus, we utilized β-galactosidase (Lac-Z) as a reporter gene to evaluate the percentage of teratocarcinoma cells in which flt-1 promoter driven transgene expression was achieved (11). Comparing the flt-1 promoter with CMV promoter-driven reporter expression, specificity of flt-1 promoter for teratocarcinoma cells was demonstrated. When teratocarcinoma cells were infected, the percentage of cells expressing the reporter was virtually equal with flt-1 promoter and with CMV promoter driven viruses. Of note, the negative cell line BEAS-2B showed no visible transgene

expression after infection with Ad5flt-1LacZ (Figure 3). These results suggest that the flt-1 promoter is active in most teratocarcinoma cells.

Transcriptional activity of cell-specific promoters typically correlates with the level of expression of the corresponding endogenous gene, thus, we hypothesized that the activity of the flt-1 promoter would correlate to the relative levels of flt-1 mRNA expressed in the tested cell lines. Such correlations could in theory be used to tailor transcriptionally targeted vectors to individual patient tumors based on determinations of the endogenous level of promoter activity in biopsy samples. Flt-1 and GAPDH mRNA levels of the cell lines were assessed by RT-PCR. Whereas similar levels of expression were seen for the housekeeping GAPDH gene in all cell lines used, flt-1 mRNA was only detected in the teratocarcinoma cell lines (Figure 4). No flt-1 mRNA could be detected even with 45 cycles PCR for the ovarian adenocarcinoma cell lysates. This correlation thus confirms that the flt-1 vector has good transcriptional fidelity in Ad vectors, and suggests assays of flt-1 mRNA could prove useful when deciding upon specific gene-based tumor therapy.

Our results demonstrate endogenous flt-1 expression in teratocarcinomas but not in ovarian adenocarcinoma cells. In addition, these data suggest that the expression of flt-1 mRNA previously reported in ovarian adenocarcinoma tumor blocks (13) was likely caused by non-teratocarcinoma cells, such as endothelial cells of the tumor vessels (Figure 4). In contrast to our findings are the results of Masood et al. (15), who found a variety of cancer cell lines positive for flt-1 expression with RT-PCR, including the ovarian adenocarcinoma cell line Hey. A possible reason for the discrepancy could relate

to the cells (eg. passage number) or be associated with the sensitivity of the RT-PCR assays used.

A question currently un-answered is the biological reason for flt-1 promoter activity in teratocarcinoma cells. Since teratocarcinomas are derived from undifferentiated, pluripotent early embryonal cells and contain cells from endo-, meso- and ectoderm (16), lack of differentiation could be a possible reason for the expression of a promoter normally active in mature endothelium. In fact, previous studies suggested cross dependance of flt-1 gene expression and differentiation status (17, 18). Therefore, we induced artificial differentiation of teratocarcinoma cells with retinoid acid and cAMP or valproic acid (19, 20), followed by infection with a luciferase expressing adenovirus. Although we could validate artificial differentiation by observing changes in CMV driven gene expression and microscopic appearance of the cells, neither experiment demonstrated an association between flt-1 expression and artificially induced differentiation (data not shown). However, both models are distinct from natural differentiation processes and thus may offer limited insight in this context.

In summary, we showed endogenous flt-1 expression in teratocarcinoma cell lines but not in ovarian, breast and cervical carcinoma cell lines. Although teratocarcinoma are rare tumors and usually treatable with chemotherapy, these findings are of potential interest since they represent the first time a promoter has been demonstrated to be active in teratocarcinoma, independent of the tissue type it originates from. The flt-1 promoter is active irrespective if the teratocarcinoma cell is derived from ovarian or testicular tissue.

Any promoters that retain fidelity when placed in the Ad genome, achieve good levels of transgene expression and have "liver off" phenotype are potentially of clinical utility. The close correlation of transgene expression and flt-1 mRNA shown here is further evidence that this promoter has the required characteristics. These data suggest that flt-1 could be useful for transcriptionally targeting teratocarcinoma, and that further evaluation in other flt-1 positive tumors is warranted.

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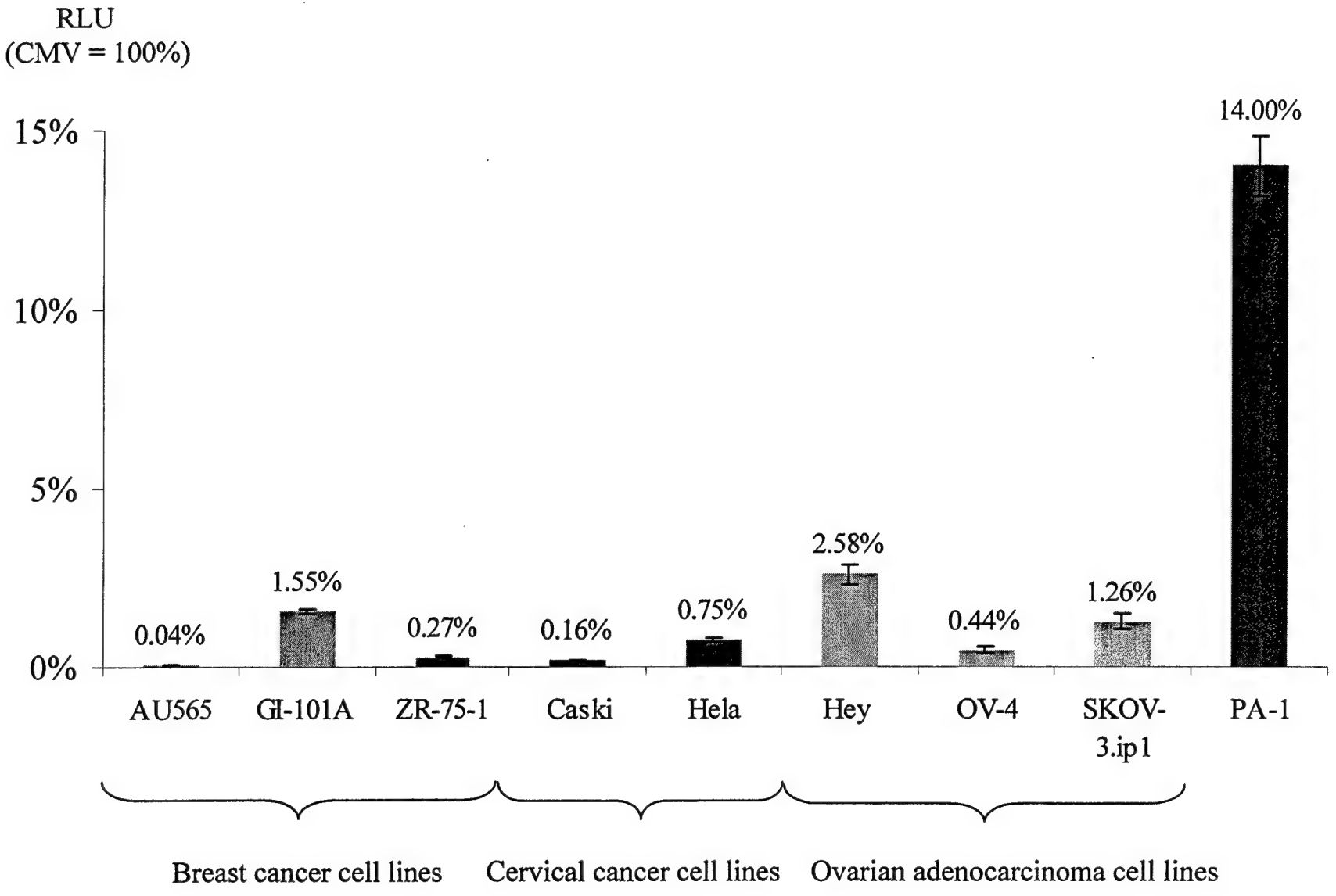
FIG. LEGENDS

Fig. 1. Adflt-1luc1 induces high marker gene expression in the PA-1 teratocarcinoma cell line, but low expression in breast, ovarian and cervical lines. Cells were infected for two hours with 5 PFU/cell of Adflt-1luc1 or AdCMVluc1. After 24 hours cells were lysed and luciferase activity was measured. Flt-1 promoter activity is presented as a percentage of CMV promoter activity. Error bars indicate standart deviation based on triplicate experiments.

Fig. 2. Adflt-1luc1 indicates high marker gene expression in all teratocarcinoma cell lines tested. PA-1 is a human ovarian teratocarcinoma cell line, NCCIT and NTERA-2 are human testicular teratocarcinoma cell lines and F9 is a murine ovarian teratocarcinoma cell line. Cells were infected for two hours with 5 PFU/cell of Adflt-1luc1 or AdCMVluc1. After 24 hours, cells were lysed and luciferase activity was measured. Flt-1 promoter activity is presented as a percentage of CMV promoter activity. Error bars indicate standart deviation based on triplicate experiments.

Fig. 3. Ad5flt-1LacZ is expressed in a high proportion of teratocarcinoma cells, but not in lung epithelial cells (BEAS-2B). Cells were infected for two hours with 500 PFU/cell of Adflt-1LacZ or AdCMVLacZ. After 24 hours, cells were fixed and stained with LacZ. Pictures were taken by bright field microscopy at 10x magnification.

Fig. 4. Flt-1 is selectively expressed in teratocarcinoma cell lines. Cells were lysed and RNA was extracted. RNA was treated with Deoxyribonulease I and RT-PCR was performed with Flt-1 and GAPDH primers, respectively. PA-1, NTERA-2 and NCCIT are teratocarcinoma cell lines. BEAS-2B is a lung epithelial cell line. Hey, OV-4 and SKOV-3.ip1 are ovarian adenocarcinoma cell lines.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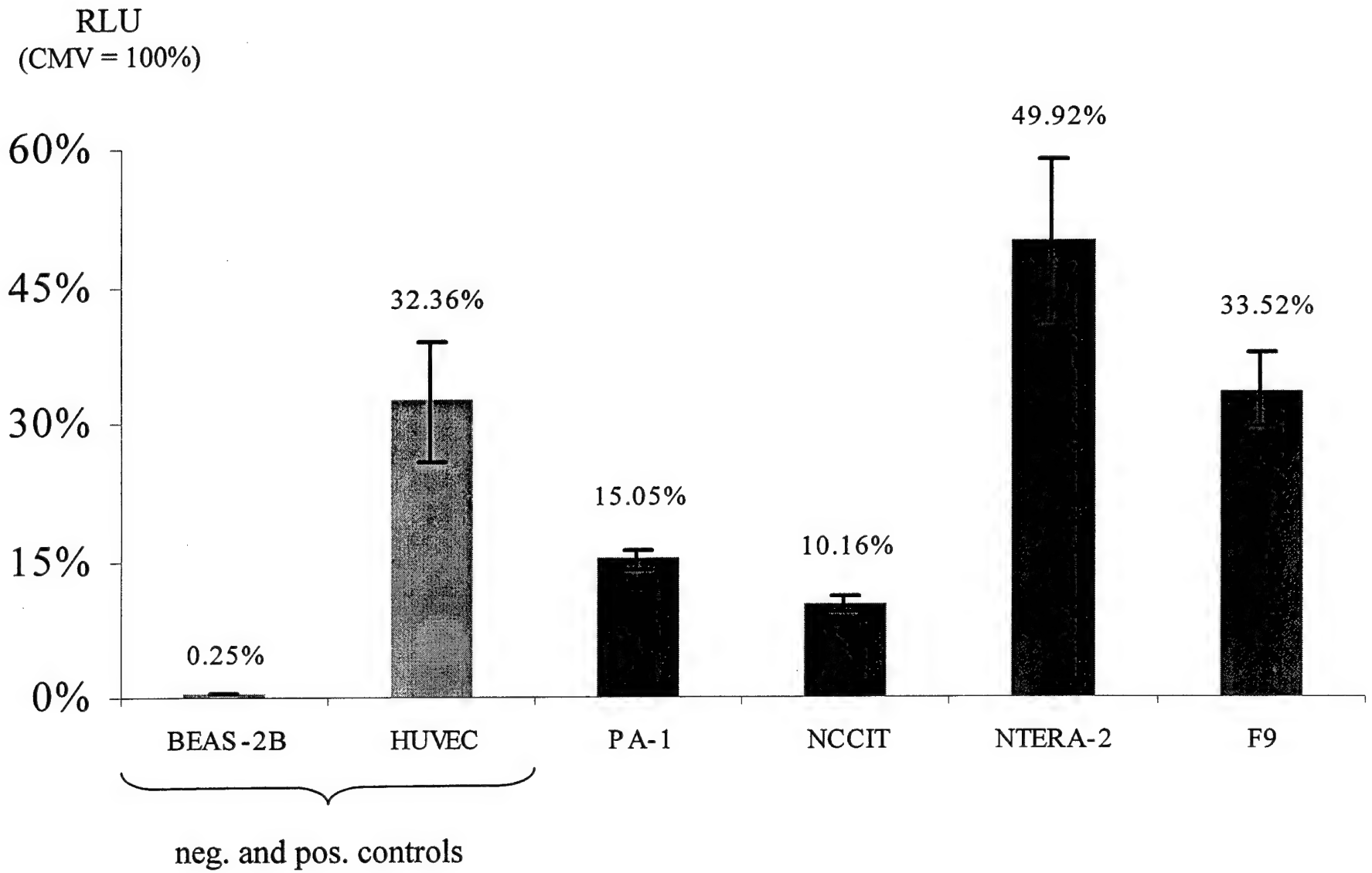
Cervical cancer cell lines
Ovarian adenocarcinoma cell lines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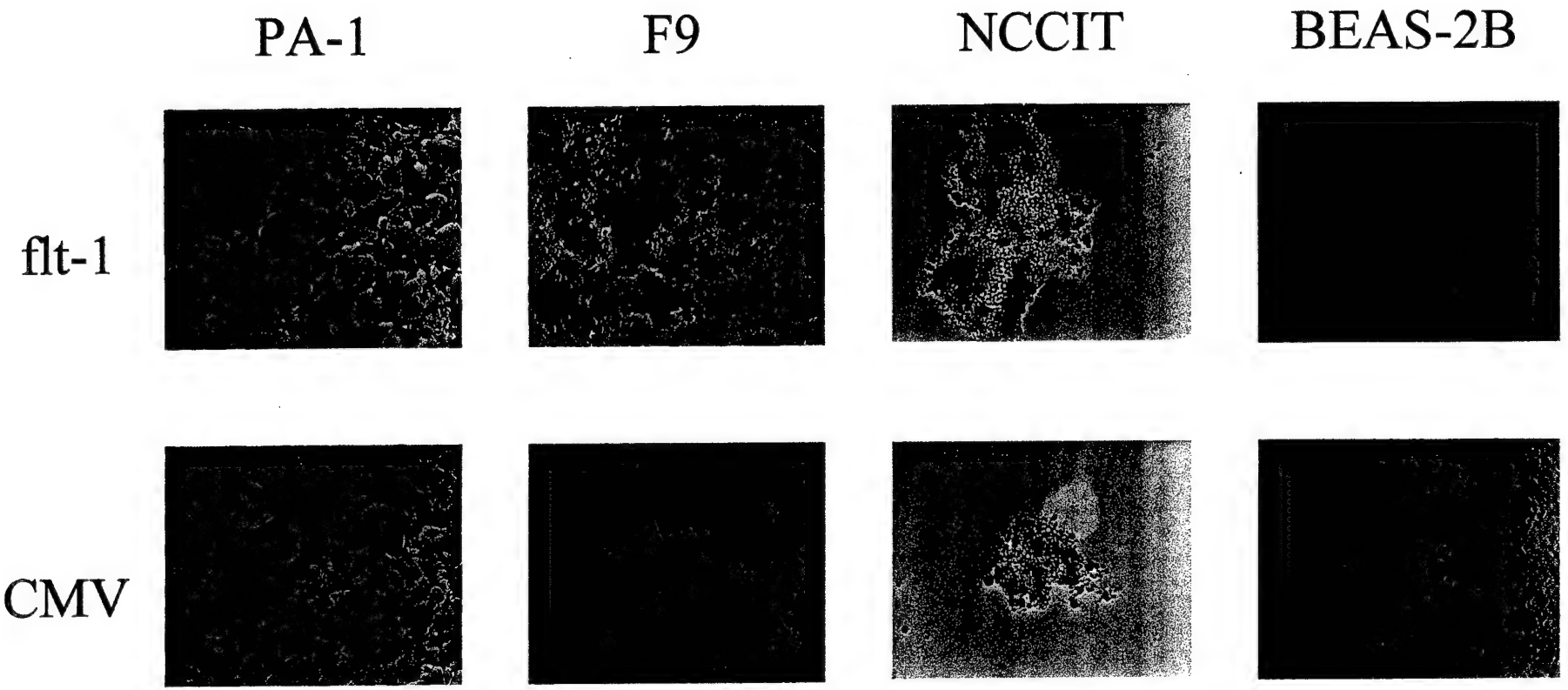
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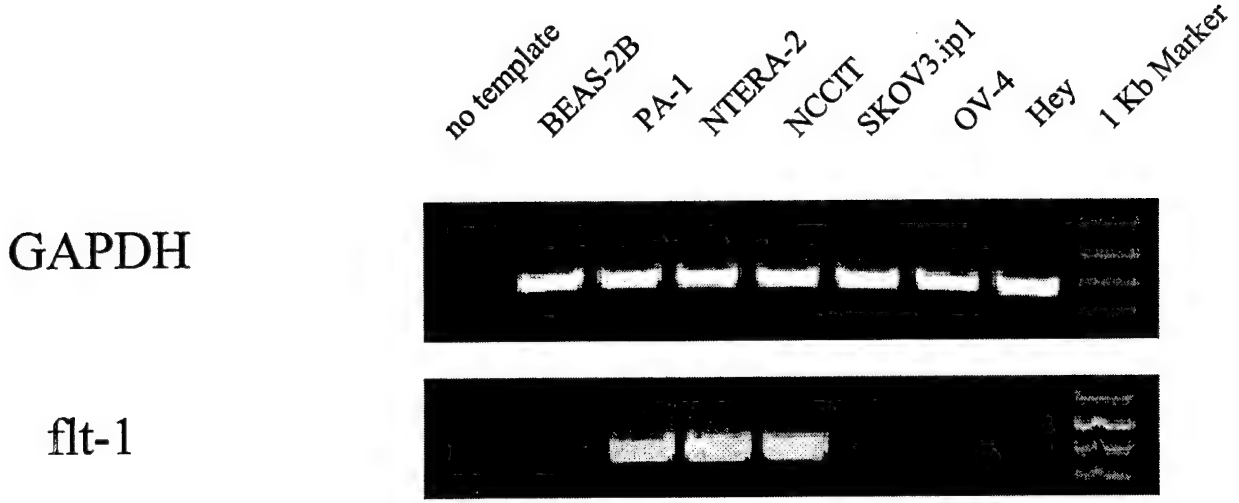
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NCCIT
SKOV3.ip1
OV-4
Hey
1 Kb Marker
GAPDH
flt-1

A Tropism Modified Oncolytic Adenovirus for Treatment of Ovarian Cancer

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Abstract

The p16-Rb pathway is defective in virtually all human cancers, including ovarian adenocarcinoma. We developed an oncolytic adenovirus, Ad5-D24RGD, which replicates selectively in cells defective in this pathway. The fiber of Ad5-D24RGD was modified with an integrin binding motif, which allows coxsackie-adenovirus receptor independent infection of cancer cells. As the expression of the requisite viral receptor is frequently low in primary tumor specimens, the fiber modification allows enhanced infectivity of cancer cells. In ovarian cancer cell lines, the virus had comparable oncolytic potential to a wild type adenovirus control. Replication of the agent in primary tumor material was shown utilizing a novel three-dimensional spheroid model based on unpassaged but purified ovarian cancer cells obtained from malignant ascites. Finally, a highly aggressive orthotopic murine model of ovarian cancer was used to test intraperitoneal administration of Ad5-D24RGD to tumor bearing animals, as would be offered in human trials. Injection of the agent resulted in eradication of intraperitoneal disease, while all control animals expired ($P<0.0001$). These results suggest that Ad5-D24RGD could be useful for treatment of ovarian cancer in humans and merit clinical evaluation.

Key Words

gene therapy, virotherapy, infectivity enhancement, conditionally replicating adenovirus, coxsackie-adenovirus receptor

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Introduction:

Ovarian adenocarcinoma is the leading cause of gynecological cancer mortality in the United States with an estimated 23,400 new cases and 13,900 deaths in 2001 (1), and the incidence is increasing (2). Due to the lack of an effective screening strategy and inconspicuous early symptoms, most patients are detected at an advanced stage, for which 5-year survival rates continue to be less than 30% (1). Typically, dissemination occurs initially intraperitoneally. Unfortunately, this group of patients have the worst prognosis. Despite aggressive surgical debulking and advances in chemotherapy, survival figures have improved minimally. Thus, there is a need for novel treatments for this patient population. The increased understanding of molecular mechanisms in neoplastic transformation and progression allows cancer to be considered a disease of mutations in genes. Therefore, it should be possible to utilize these molecular differences between normal and malignant tissue to develop agents specific for cancer cells.

Conditionally replicating adenoviruses (CRADs) take advantage of tumor specific changes which allow preferential replication of the agent in tumor cells (3,4). Replication causes oncolytic death of the cell and subsequent release of virions, and subsequent infection of surrounding cells, resulting in efficient tumor penetration and amplification of effect. Importantly, the anti-tumor effect of CRADs is determined by the capability of the agent to infect tumor cells (5-7). Unfortunately, recent evidence suggests that the expression level of the coxsackie-adenovirus receptor (CAR) is highly variable and often low on primary ovarian cancer cells (8-12). This is concerning, as CAR expression appears to be the major factor determining infectivity with the most commonly used adenovirus (Ad) serotypes, including the serotype 5 used here (13-17). Although the function of CAR is not well understood, it may be linked to

adhesion and could have tumor suppressing effect (18,19). Also, CAR expression could correlate inversely with the stage of the tumor (18,20). Furthermore, preliminary evidence suggests that over-activity of the *RAS-MAPK* pathway, commonly seen in any tumor type, could cause CAR down-regulation (20). In addition to ovarian cancer, frequent CAR-deficiency has been shown for various other tumor types, and could be a ubiquitous phenomenon (reviewed in Ref. 21). As most normal epithelial tissues express CAR, use of untargeted Ads could result in transduction of mainly non-tumor cells. Nevertheless, even first generation CRADs have shown some clinical utility (21). This suggests that, if infectivity and replicativity of the agents can be improved, further improvements in clinical efficacy could be gained.

In this study, we have used a replication competent recombinant Ad (Ad5-D24RGD), which has a 24 bp deletion in the constant region 2 (CR2) of the *E1A* gene (22). This domain of the E1A protein is responsible for binding the retinoblastoma tumor suppressor/cell cycle regulator protein (Rb), which allows Ad to induce S-phase entry (23). Therefore, viruses with this type of deletion are reduced in their ability to overcome the G1-S checkpoint and replicate efficiently only in cells where this interaction is not necessary, *eg.* tumor cells defective in the *Rb-p16* pathway (24,25). Appropriately, this pathway seems to be inactive in almost all human tumors (26), including ovarian cancers (27-30). To circumvent the frequent CAR-deficiency in primary ovarian cancers, the fiber of Ad5-D24RGD was modified by incorporating an integrin binding RGD-4C motif into the HI-loop. This infectivity enhancement has been shown to dramatically increase transduction of ovarian cancer cells (8,10,12). As most ovarian cancer patients present with ascites, it is important to note that the RGD-4C modification allows partial escape from neutralizing antibodies regularly present in the ascites (12,31).

Using this novel infectivity enhanced CRAD, we demonstrate replication in and oncolysis of ovarian cancer cells. Further, we demonstrate replication of the agent in primary ovarian cancer spheroids, a novel three-dimensional model of human cancer. Finally, the agent displayed impressive therapeutic efficacy in an orthotopic murine model of ovarian cancer. These results suggest that Ad5-D24RGD could be a highly useful agent for testing in human trials.

Materials and Methods:

Cell culture. Hey, OV-4 and SKOV3.ip1 ovarian adenocarcinoma cell lines are kind gifts from Dr. Timothy J. Eberlein (Harvard Medical School, Boston, MA), Dr. Judy Wolf and Dr. Janet Price (both M.D. Anderson Cancer Center, Houston, TX). The ovarian teratocarcinoma cell line PA-1 and the human lung adenocarcinoma cell line A549 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The 293 human transformed embryonal kidney cell line was purchased from Microbix (Toronto, Canada). All cell lines were maintained in a humidified 37°C atmosphere containing 5% CO_2. Hey was grown in RPMI-1640 (Mediatech, Herndon, VA) whereas A549, 293, OV-4, PA-1 and SKOV3.ip1 were grown in Dulbecco's Modified Eagle's Medium/F12 (Mediatech, Herndon, VA), both supplemented with 2 nM L-Glutamine, 100 IU/ml Penicillin, 25 µg/ml Streptomycin and 10% fetal bovine serum (FBS, Hyclone, Logan, UT). Infections were performed in growth medium (GM) with 2% FBS.

Viruses. Ad5-D24RGD was created using a shuttle vector containing a 24 bp deletion in the CR2 region (the Rb binding site) of *E1A* (22). The shuttle was co-transformed into *E. coli* cells for homologous recombination with pVK503, a rescue plasmid containing the RGD-4C modification of the fiber. This resulted in a plasmid with the full length Ad genome with the 24 bp deletion. The plasmid was digested with *PacI* to release the Ad5-D24RGD genome (Figure 1) for transfection into packaging cells. Propagation was performed on A549 cells. Ad5wtRGD was created by digesting pVK503 with *PacI,* followed by transfection into 293 cells, resulting in an otherwise wild type adenovirus but with the RGD-4C modification in the fiber. This virus is expected to display replication similar to wild type as the early genes are unmodified. Ad5wtRGD was created to allow normalization for infectivity when comparing the replicativity

of wild type virus with Ad5-D24RGD. Ad5wtRGD and Ad5lucRGD (8) were propagated on 293 cells. All viruses were purified with double CsCl gradients using standard methods.

The viruses were titered for viral particles (VP) using standard methods based on spectrophotometry. Functional titer (plaque forming units, PFU) was determined with plaque assay with an initial overnight infection of 293 cells. The viruses had the following titers: Ad5wtRGD 1.0 x 10^{12} VP/ml, 2.2 x 10^{10} PFU/ml, 45 VP/PFU; Ad5lucRGD 9.7 x 10^{11} VP/ml, 1.9 x 10^{10} PFU/ml, 51 VP/PFU; Ad5-D24RGD 2.6 x 10^{12} VP/ml, 5.2 x 10^{10} PFU/ml, 50 VP/PFU. The presence of the *E3* region and the RGD-4C modification were confirmed with PCR (primers: E3L2: 5'-CCTGAAACACCTGGTCCACT-3', E3R2: 5'-GCCACAGTTAGGGCTTCTGA-3'; FiberUp: 5'-CAAACGCTGTTGGATTTATG-3'; FiberDown: 5'-GTGTAAGAGGATGTGGCAAAT-3'). The presence of the 24 bp deletion in *E1A* and the absence of wild type *E1A* was confirmed with PCR (primers: D24L1: 5'-GTCCGGTTTCTATGCCAAAC-3', D24R1: 5'-TCACCCTCTTCATCCTCGTC-3') followed by sequencing.

Crystal violet cell killing assay. Hey, SKOV3.ip1, PA-1 and OV-4 cells were plated on day 1 at 500,000 cells per well on 6-well plates in 3 ml GM containing 5% FBS. On day 2, cells were washed once with 2 ml Phoshate Buffered Saline (PBS) and infected with 0, 0.1, 1 or 10 VP/cell for 1 h in 500 µl 2% GM on a rocker. Afterwards, cells were washed again with 2 ml PBS, and 2 ml GM (5% FBS) was added per well. Every other day the full volume of GM was changed. On day 10 (PA-1), day 14 (Hey and SKOV3.ip1) or day 17 (OV-4), respectively, plates were washed twice with PBS, cells were fixed for 10 min at room temperature in 10% buffered formalin and stained with 1% crystal violet in 70% ethanol for 20 min.

Protein concentration based oncolysis assay. On day 1, Hey, SKOV3.ip1, PA-1 and OV-4 cells were plated at 15,000 cells per well on 96-well plates in 200 µl GM containing 10% FBS. On day 2 cells were infected with 0, 0.1, 1 or 10 VP/cell for 1 h in 100 µl 2% GM on a rocker. Afterwards, cells were washed with 200 µl PBS and then covered with 200 µl GM (5% FBS). Every other day, 50% of the full volume of the GM was changed. On day 8 (PA-1), day 15 (Hey and SKOV3.ip1) or day 16 (OV-4), plates were washed twice with PBS, cells were lysed with 100 µl lysis buffer (Reporter Lysis Buffer, Promega, Madison, WI) and freeze-thawed once. Protein concentration was measured with the DC Protein Asssay system (Bio-Rad Laboratories, Hercules, CA).

Quantitative PCR. Ovarian adenocarcinoma primary cells were purified from malignant ascites samples obtained from patients undergoing a procedure for ovarian cancer at the University of Alabama Hospital. The purification protocol is based on a mouse anti-human-TAG-72 antibody and anti-mouse-IgG coated magnetic beads and has been described previously (32). Typically, the purification yields nearly 100% cancer cells. Analysis and creation of spheroids from primary ovarian carcinoma cells is detailed elsewhere (33). Briefly, purified unpassaged cells were incubated overnight at 37 °C with 5% CO_2 in a 3% agar coated flask on a rocker to form spheroids, *ie.* three-dimensional clusters of cells. The spheroids were resuspended and infected with 1000 VP of Ad5-D24RGD per cell. One hour after infection, FBS was added to achieve 10% FBS. Twenty-four hours after the infection, the spheroids were divided into five equal aliquots of 1×10^5 cells and each was resuspended in 200 µl of GM. For five consecutive days after infection, an aliquot of spheroids was frozen at –20 °C. Afterwards, DNA was extracted with QIAamp DNA Mini Kit (Qiagen Inc., Valencia, CA), and quantitative PCR for the E1 gene was performed with LightCycler methodology (Roche Diagnostics GmbH,

Mannheim, Germany) as described previously (12). In order to display the negative control, readings below the assay's detection limit were set as 1 and other results are displayed relative to this control.

Therapeutic ovarian carcinoma model. CB17 SCID mice (n=11/group) were injected with 1 x 10^7 SKOV3.ip1 cells intraperitoneally (ip.) on day 0. On days 4, 5 and 6, mice were injected ip. with 1 x 10^{10} or 5 x 10^8 VP of Ad5-D24RGD, Ad5lucRGD (non-replicative control) or no virus. The virus was diluted with Opti-MEM into 1 ml in each case. In accordance with Institutional Animal Care and Use Committee guidelines, mice were inspected daily and euthanasia was performed in case of discomfort or distress. Survival data was plotted into a Kaplan-Meier curve and, using the LIFETEST procedure in SAS v.8.2, the Ad5-D24RGD groups were compared to the other groups with the log-rank test (34). The distribution of the data best fit the Weibull model, which was utilized for individual comparisons between the Ad5-D24RGD groups and controls, using the chi-square test of SAS v.8.2 LIFEREG procedure (35).

Results:

Ad5-D24RGD replicates in and kills ovarian carcinoma cells. In all cell lines, the crystal violet based cell killing assay showed replication of Ad5-D24RGD and Ad5wtRGD, which was included as a positive control (Figure 2a). Replication resulted in oncolytic death of cells, as seen by the loss of monolayer in the crystal violet staining. In the adenocarcinoma lines (Hey, SKOV3.ip1 and Ov-4), the CRAD replicated to similar degree as the virus containing the wild type early genes. For PA-1, a teratocarcinoma line, the oncolytic effect of Ad5wtRGD was slightly stronger than Ad5-D24RGD. Ad5LucRGD was included as a non-replicative control and did not cause oncolysis.

The crystal violet findings were confirmed with a more quantitative assay based on protein concentration, which reflects the amount of cells left after replication and oncolysis (Figure 2b). When 10 VP/cell of Ad5-D24RGD was used for infection of Hey, SKOV3.ip1, PA-1 and OV-4 cells, 11.2%, 46.2%, 73.0% and 46.7% of cells remained alive (as compared to uninfected wells). For Ad5wtRGD, the positive control, the results were similar (13.7%, 22.7%, 28.0%, 51.9%). Therefore, Ad5-D24RGD has similar oncolytic potential to a wild type virus.

Replication of Ad5-D24RGD in ovarian cancer primary cell spheroids. Ovarian cancer primary cells spheroids provide a useful three-dimensional model for assessing replicativity of CRADs. Perhaps more importantly, they provide a convenient means of maintaining primary cells alive in culture, without the confounding effect caused by clonal selection pressure involved in passaging and adaptation to cell culture. Spheroids were collected 1 thru 5 days after infection and quantitative PCR was performed to detect viral copies (Figure 3). One day after infection, 1.13 copies/well were detected, and the number grew exponentially to 1,036 – 19,336 – 402,000

– 4,296,000 copies on days 2 - 3 - 4 - 5. Thus, Ad5-D24RGD infects and replicates in primary unpassaged ovarian cancer cells.

Therapeutic effect of Ad5-D24RGD in an intraperitoneal model of ovarian cancer. We utilized a well established murine ovarian cancer model based on ip. inoculation of SKOV3.ip1 cells. Three ip. doses of 1 x 10^{10} VP/day of Ad5-D24RGD, the non-replicative Ad5lucRGD or no virus were injected after establishing ip. tumors (Figure 4a). The median survival of mice was 64, 45 and 36 days, respectively and mean survival times for Ad5lucRGD and no virus were 45.7 and 37.6 days, respectively. Statistical analysis with the log-rank and chi-square tests indicated that survival was significantly better in animals treated with Ad5-D24RGD ($P<0.0001$).

A smaller dose of the viruses (5 x 10^8 VP/day ip. for three days) was also investigated (Figure 4b). The median survival was not reached for Ad5-D24RGD. For Ad5LucRGD and no virus, the median survival was 40 and 36 days and means were 41.9 and 37.6 days, respectively. All mice in the control groups expired before day 60. In the group of mice treated with 5 x 10^8 VP of Ad5-D24RGD, all mice survived until at least day 61. The log-rank and chi-square tests confirmed that survival was significantly better in animals treated with Ad5-D24RGD ($P<0.0001$).

Interestingly, none of the mice treated with Ad5-D24RGD showed any evidence of intraperitoneal disease after treatment. Instead, many developed subcutaneous tumors at the site where the ip. tumor cell injection had been performed, which eventually necessitated sacrificing the animals. All animals in the control groups expired or were sacrificed due to intraperitoneal tumor growth.

Discussion:

Treatment of malignancies resistant to traditional modalities requires novel approaches. Gene therapy applications where viral or non-viral vehicles are used for gene transfer, have shown tremendous promise in preclinical studies, but inefficient tumor transduction has often precluded significant clinical benefit. To address this crucial aspect, CRADs are emerging as a powerful way to improve tumor penetration and amplify the delivered dose (3,4).

However, another obstacle has become evident. Unlike normal epithelial tissues and many cell lines, primary tumors seem to express variable and often very low amounts of the requisite receptor, CAR. As CAR levels appear to be the major factor determining binding and subsequent infectivity, low CAR may translate into low tumor transduction and low CRAD efficacy (see Introduction). The relationship between infectivity and oncolytic potency has been confirmed in experimental models (5-7). Therefore, it seems likely, that to achieve significant anti-tumor effect, it is necessary to direct gene therapy agents to targets more prevalent in tumor tissue. In this study, we have utilized the well characterized RGD-4C motif, which binds the $\alpha_v\beta$ group of integrins (8,36), constitutively expressed and frequently overexpressed in various types of cancer, including ovarian adenocarcinomas (9,37,38). This infectivity enhancement allows dramatically increased transduction of primary ovarian cancer cells (8,10,12).

All ovarian cancer gene therapy trials published thus far have relied on intraperitoneal inoculation of the agent. This is due to the disease usually presenting at an advanced stage, typically with peritoneal metastasis, which has the worst prognosis (1). Intraperitoneal administration and re-administration can be conveniently achieved with a catheter and the peritoneal cavity allows a degree of compartmentalization, which may have contributed to the excellent safety profile observed in Phase I studies (21). The peritoneal cavity could also allow

effective dissemination of a CRAD, resulting in good transduction of intraperitoneal tumor masses. However, these patients usually have malignant ascites, which contains anti-Ad neutralizing antibodies (31,39). It is important to note that the RGD-4C modification of the fiber allows Ad to partially escape pre-existing anti-Ad antibodies, which could improve transduction of tumor tissue (12,31).

The replicative selectivity of Ad5-D24RGD (Figure 1) is based on incapability in binding Rb and therefore replication is expected to occur preferentially in cells where S-phase induction is not required, such as cells defective in the Rb-p16 pathway. This pathway may be faulty in all human cancers (26), including ovarian adenocarcinomas (27-30). Previously, it has been shown that replication of CR2 deleted viruses is attenuated in non-proliferating normal cells (24,25). Interestingly, abrogation of replication was also demonstrated when Rb was reintroduced into otherwise permissive cells (24).

In this study, we show replication of Ad5-D24RGD in ovarian cancer cell lines (Figure 2). The assays used here measure both replication and subsequent oncolysis, as the readout corresponds with the amount of live cells remaining. Impressively, the potency of Ad5-D24RGD was very similar to Ad5wtRGD, which is wild-type except for the fiber modification. This virus was chosen as the positive control to avoid bias due to the differences in infectivity (and subsequent oncolytic potency) caused by fiber modifications. These findings are in accord with other investigators', who have studied the effect of the CR2 deletion on replicativity and oncolytic potency (25). In cancer cell lines, a CR2 deleted virus demonstrated similar to superior replicativity when compared to wild type Ad (25). Also, the CR2 deleted virus was compared to the E1B55K deleted dl1520 virus (also called ONYX-015 and CI-1042) (40,41), which has been used extensively for treatment of patients (42). Although there are some promising results in

combination with chemotherapy (43), the evidence showing replication of dl1520 in tumor tissue has been anecdotal and the single agent clinical efficacy has been marginal (42). This could be due to the replicativity of this agent, which is low even *in vitro* (40,41,44). In contrast, as the CR2 deletion does not seem to affect replicativity to a significant degree, it will be interesting to see if this leads to higher therapeutic efficacy.

Previous studies with Ads have suggested that there is a disconnect between transduction efficacy in preclinical versus human studies (45,46). An important reason for this may be the highly variable expression of CAR in primary tissue. Therefore, we and others have used unpassaged and purified primary tumor material as our most stringent template for testing novel approaches (8-10,12,32,47). A problem with primary cells is that they tend not to stay alive very long *in vitro*, and thus analysis of replication and oncolysis is difficult. We have developed an ovarian primary cell spheroid model, which allows purified cancer cells to stay alive for at least a month (33). Also, the three dimensional structure of spheroids may resemble *in vivo* tumors better than two-dimensional cell line cultures. Here, we tested the replication of Ad5-D24RGD on spheroids and observed an exponential increase in viral particles as a function of time (Figure 3). This suggests that Ad5-D24RGD can infect and replicates in primary ovarian cancer cells.

The ultimate preclinical test of an experimental therapeutic is an orthotopic animal model. Here, we utilized a murine model of ovarian cancer, and performed intraperitoneal injections of the virus, as would be offered in a human trial (Figure 4). All mice treated with Ad5-D24RGD showed no evidence of intraperitoneal disease. However, many mice relapsed with subcutaneous tumors. This could have been caused by a small number of tumor cells contaminating the needle tract during injection of cells. The virus probably had little access to the subcutaneous tissue and therefore could not eradicate these cells. It should be noted that no

cures or long term survival has previously been reported for this aggressive model of ovarian cancer (48,49).

With the lowest viral dose used, we saw 100% survival up to 61 days, when all animals in the control groups had expired (Figure 4). The only other CRAD that has been used for treatment of an ovarian cancer animal model is dl1520 (50). Two days after ip. inoculation of A2780 tumors, 1 x 10^9 PFU (ca. 2 x 10^{10} VP) was injected daily for 5 days, resulting in 40% of mice showing no evidence of ip. disease. Five doses of 4 x 10^8 VP of dl1520 ip. into mice bearing OvCAR3 tumor resulted in resolving of ip. tumors in 4/4 cases, but follow-up was only 42 days. Although direct comparison is difficult due to different experimental design, the *in vivo* efficacy of Ad5-D24RGD could compare favorably to dl1520.

The dose used here was 5 x 10^8 VP daily for three days, which would equal ca. 1 x 10^{12} VP for a 60 kg human (w/w). dl1520 has been administered to humans ip. with doses ranging from a daily dose of 1 x 10^{11} to 1 x 10^{13} VP for 5 consecutive days (42). Although the final data are currently unavailable, the preliminary results suggest that patients with bulky tumors (>2 cm) experienced dose limiting side effects at 1 x 10^{12} VP x 5, while patients with non-bulky tumors tolerated 1 x 10^{13} VP x 5 without toxicity. While comparisons between mouse and human data should be avoided, since human Ads do not replicate in murine tissues to any significant degree, these figures suggest that the oncolytic potency of Ad5-D24RGD is sufficient to merit a human Phase I trial.

Recently, it has been demonstrated that gene therapy is a feasible way of achieving clinical benefits in patients (51-53). Interestingly, these findings have come from the seemingly unrelated fields of genetic disease on one hand and acquired vascular diseases on the other. What these success stories have in common is the rational approach investigators have had in

developing the gene delivery tools. Thus, the clinical breakthroughs were based on advances in vector development. The approach described in this paper combines two promising fields, replication competent viruses and infectivity enhancement. Together, these powerful means to increase tumor transduction could help achieve similar breakthroughs in the field of cancer gene therapy.

In conclusion, we have utilized a novel replication competent agent, Ad5-D24RGD, for treatment of ovarian cancer models. The virus has been infectivity enhanced for CAR independent infection of tumor cells. We observed replication and oncolytic potency similar to a wild type control virus (both viruses were fiber modified). Utilizing a highly novel three dimensional spheroid model, we detected exponential increase in the amount of Ad5-D24RGD gene copies suggesting efficient replication. Finally, we used a stringent orthotopic murine model of ovarian cancer and saw significant improvement in survival of the animals. In fact, all animals displayed complete eradication of ip. disease. These results suggest that Ad5-D24RGD could be an effective agent for treatment of ovarian cancer, and the toxicity should be evaluated in a Phase I trial. Also, considering that Rb-p16 pathway abnormalities in tumors seem to be ubiquitous (26), the agent could prove useful for other types of tumors as well.

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Figure legends

Figure 1

Schema of Ad5-D24RGD. Ad5-D24RGD is a conditionally replicating adenovirus with a 24 bp deletion in the Rb binding site of E1A CR2 resulting in selective replication in cells deficient in the Rb/p16 pathway. Most human tumors, including ovarian cancers, are defective in this pathway. In addition, the fiber knob has been modified to incorporate an RGD-4C motif, which allows CAR-independent transduction of tumor cells.

Figure 2

a) Replication of Ad5-D24RGD kills ovarian carcinoma cells. Cells were infected for one hour with 0, 0.1, 1 or 10 VP/cell of Ad5lucRGD (non-replicative negative control), Ad5-D24RGD or Ad5wtRGD (positive control for replication). On day 10 (PA-1), day 14 (Hey and SKOV3.ip1) or day 17 (OV-4), respectively, plates were washed and stained with crystal violet to detect remaining cells. Each experiment was performed in triplicate wells. **b)** Ad5-D24RGD causes oncolysis of ovarian carcinoma cells. Oncolysis assay based on protein concentration. Cells were infected for one hour with 0, 0.1, 1 or 10 VP/cell of Ad5lucRGD (non-replicative negative control), Ad5-D24RGD or Ad5wtRGD (positive control for replication). On day 8 (PA-1), day 15 (Hey and SKOV3.ip1) or day 16 (OV-4), respectively, protein concentration was determined. The protein concentration of cells infected with 0 VP/cell was set at 100%. Error bars indicate +/- 1 SD resulting from quadruplicate experiments.

Figure 3

Replication of Ad5-D24RGD in ovarian cancer primary cell spheroids. Spheroids represent a three-dimensional model of human cancer and increase viability of primary cells without passaging. Ovarian adenocarcinoma cells were purified from malignant ascites and spheroids were allowed to form in agarose coated flasks. The spheroids were then infected and aliquots were collected on five consecutive days. Virus particles were detected with quantitative PCR for the E1 gene. E1 copy number from spheroids infected with 0 VP/cell was set as 1.

Figure 4

Therapeutic effect of Ad5-D24RGD in an intraperitoneal (ip.) model of ovarian cancer. Intraperitoneal tumors were induced by inoculation of 1×10^7 SKOV3.ip1 cells on day zero. Four, five and six days later, Ad5-D24RGD, Ad5lucRGD (non-replicative control) or no virus were injected ip. at doses of **a)** 1×10^{10} VP/day or **b)** 5×10^8 VP/day. Survival was improved in mice treated with Ad5-D24RGD (* $P<0.0001$) and all of these mice displayed eradication of intraperitoneal tumors.

Figure 1

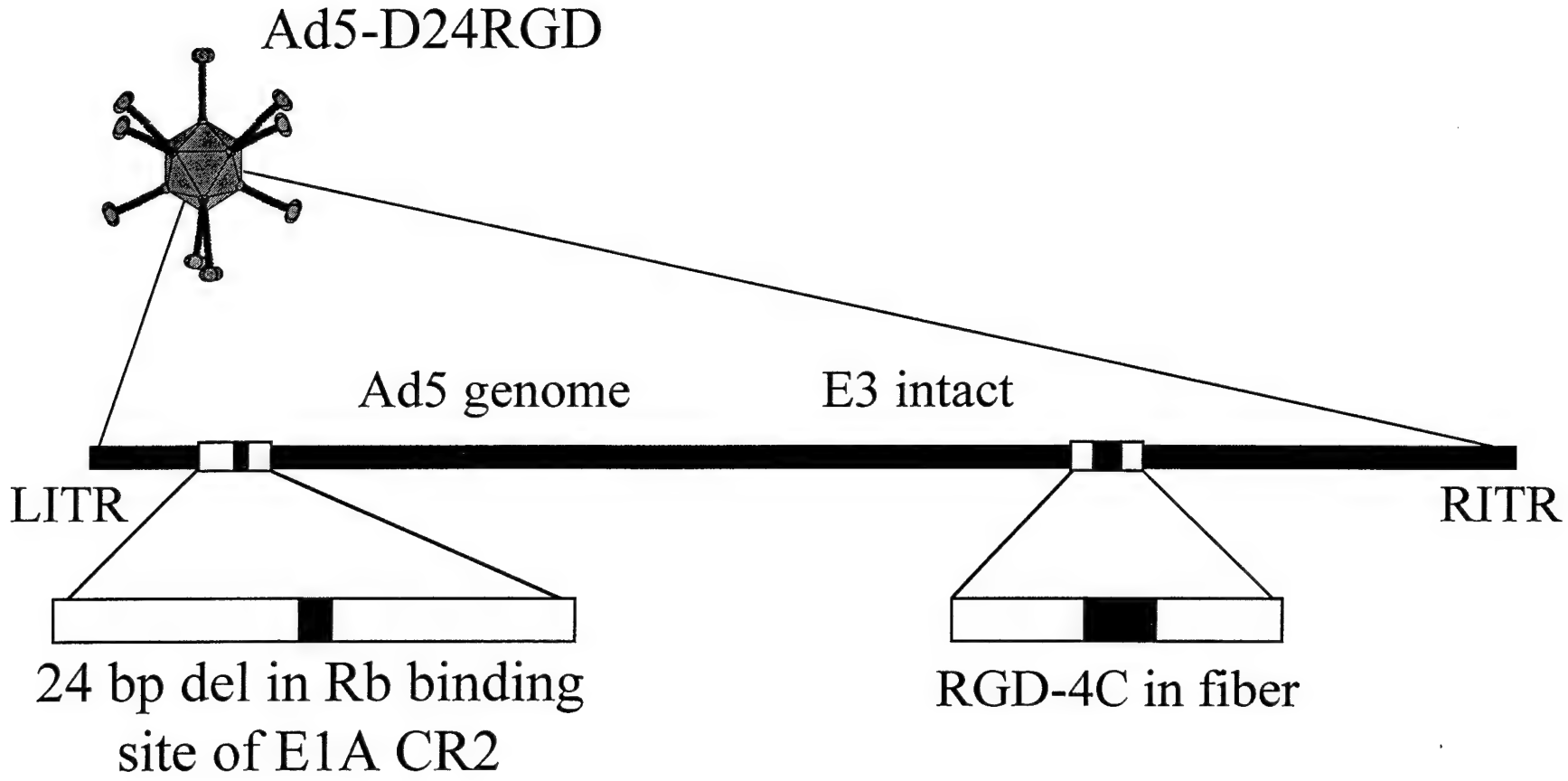
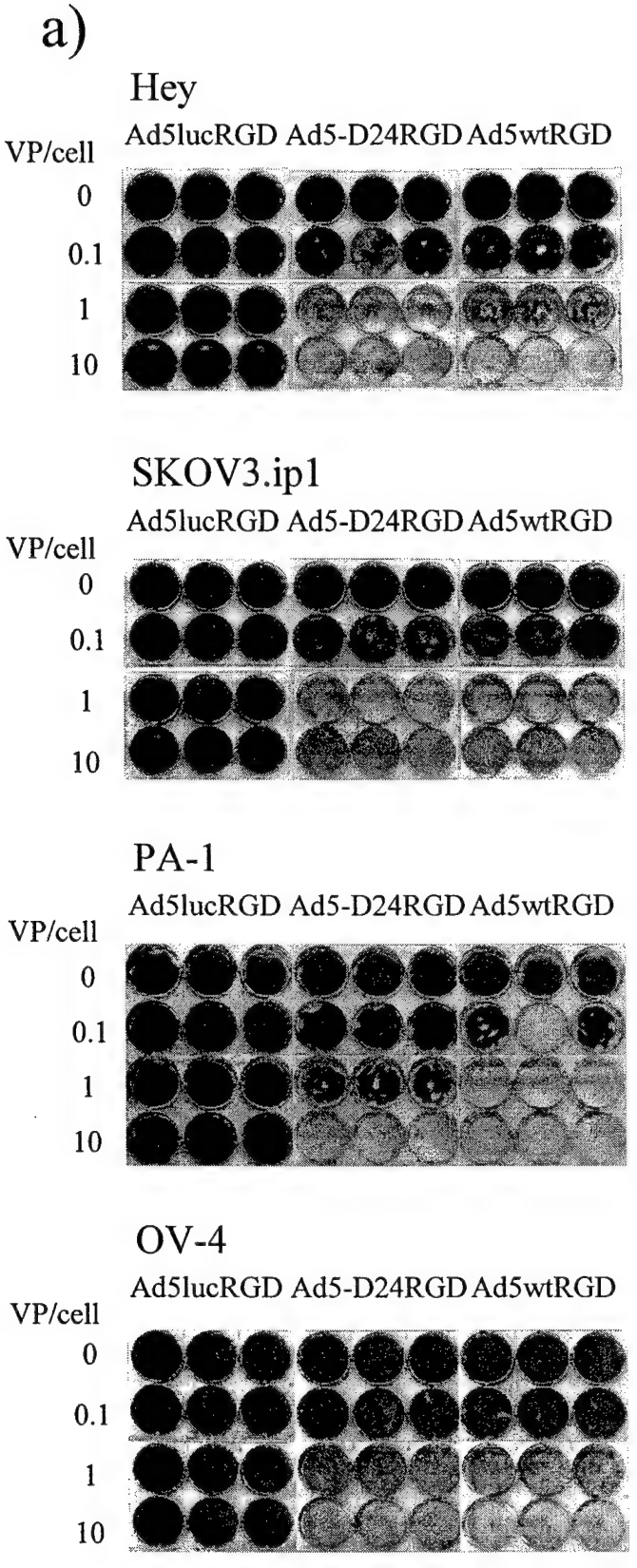

Ad5-D24RGD
Ad5 genome
E3 intact
LITR
RITR
24 bp del in Rb binding site of E1A CR2
RGD-4C in fiber

Figure 2

a)


Hey
Ad5lucRGD Ad5-D24RGD Ad5wtRGD
VP/cell
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SKOV3.ip1
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PA-1
Ad5lucRGD Ad5-D24RGD Ad5wtRGD
VP/cell
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0.1
1
10
OV-4
Ad5lucRGD Ad5-D24RGD Ad5wtRGD
VP/cell
0
0.1
1
10

b)

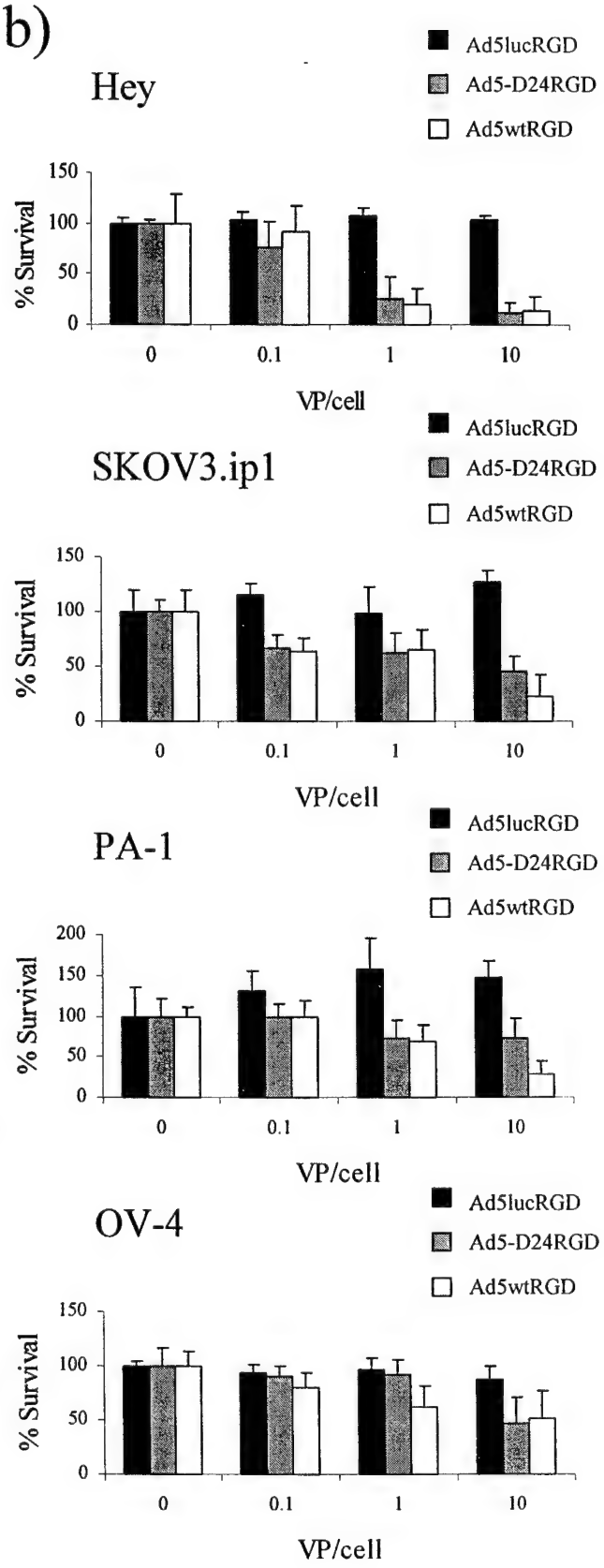

Hey
Ad5lucRGD
Ad5-D24RGD
Ad5wtRGD
% Survival
150
100
50
0
0
0.1
1
10
VP/cell
SKOV3.ip1
Ad5lucRGD
Ad5-D24RGD
Ad5wtRGD
% Survival
150
100
50
0
0
0.1
1
10
VP/cell
PA-1
Ad5lucRGD
Ad5-D24RGD
Ad5wtRGD
% Survival
200
150
100
50
0
0
0.1
1
10
VP/cell
OV-4
Ad5lucRGD
Ad5-D24RGD
Ad5wtRGD
% Survival
150
100
50
0
0
0.1
1
10
VP/cell

Figure 3

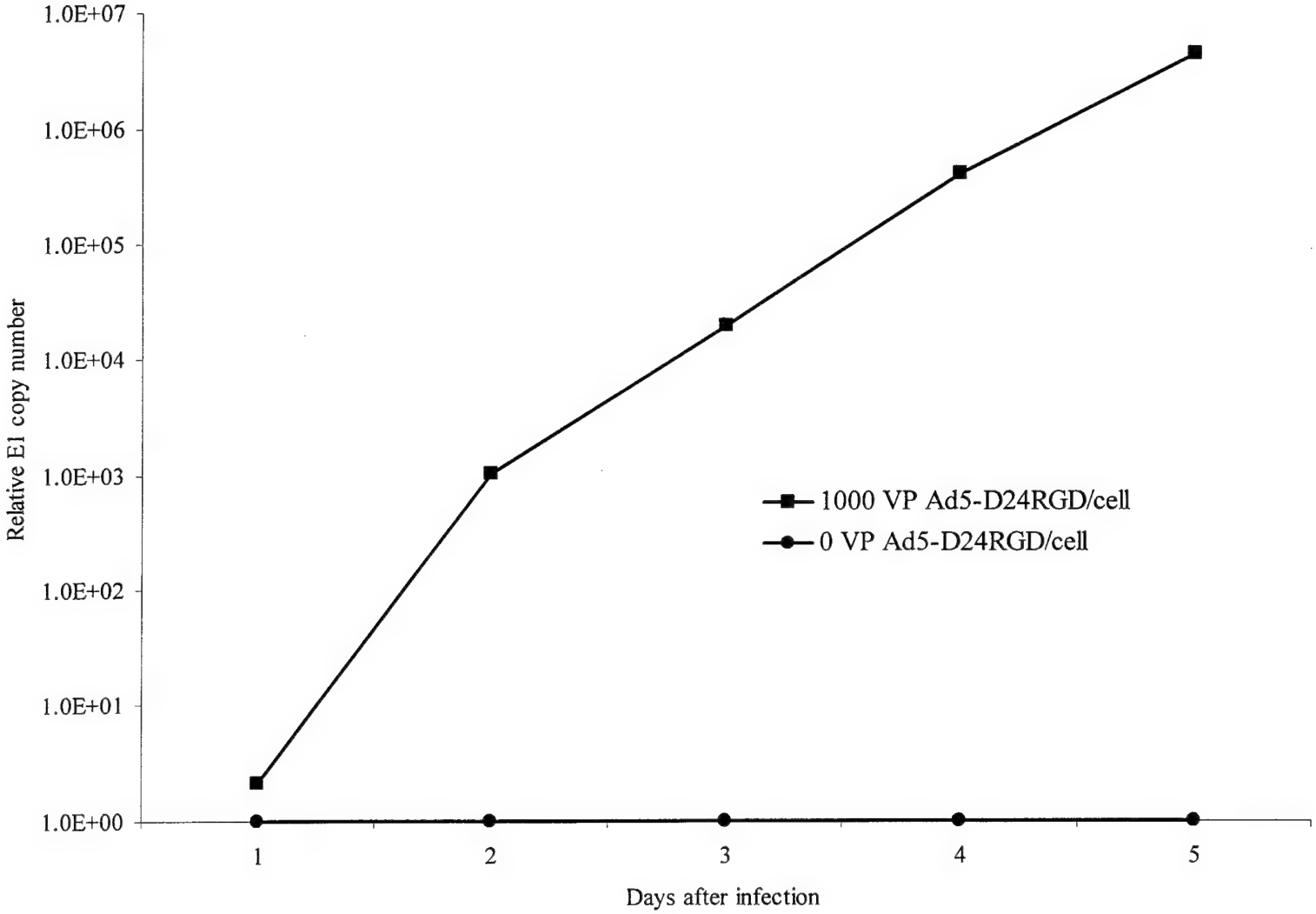
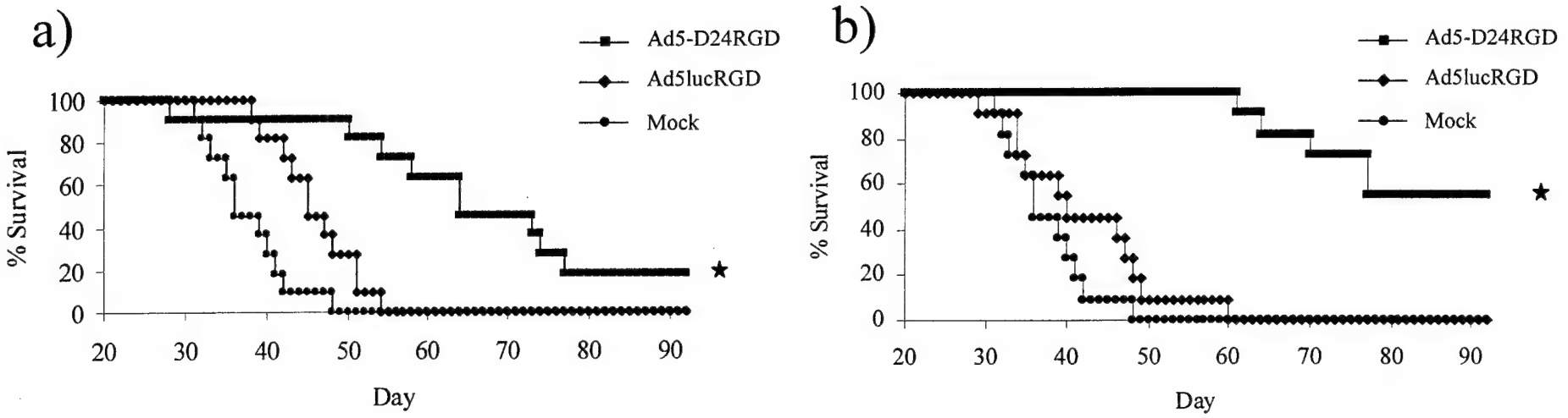

1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
Relative E1 copy number
1
2
3
4
5
Days after infection
1000 VP Ad5-D24RGD/cell
0 VP Ad5-D24RGD/cell

Figure 4


a)
Ad5-D24RGD
Ad5lucRGD
Mock
100
80
60
40
20
0
% Survival
20
30
40
50
60
70
80
90
Day
b)
Ad5-D24RGD
Ad5lucRGD
Mock
100
80
60
40
20
0
% Survival
20
30
40
50
60
70
80
90
Day

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Adenovirus Knob Chimerism Enhances Oncolytic Replication In Tumor Cells That Are Refractory To Adenovirus Serotype 5 Infection

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Running title:

Knob chimerism enhances oncolytic replication

Abstract

Adenovirus (Ad) serotype 5 (Ad5) continues to be the predominant Ad vector used for cancer gene therapy, however an increasing number of tumor types have been reported to be relatively refractory to Ad5 infection due to low expression of the native Ad5 receptor, CAR. The CAR deficiency in some tumor cells has led to the development of CAR-independent transduction strategies including the introduction of heterologous ligand sequences into the virus fiber gene and immunological and chemical modifications of the capsid proteins. Alternatively, native Ad5 tropism can be modified by substituting the knob region from other Ad serotypes, such as Ad type 3 (Ad3), with that of the Ad5 knob region. To date however, the effect(s) of tropism modification on the oncolytic capacity of replicating Ad has not been fully evaluated. Therefore, two replication-competent viruses were compared in this study: an Ad5 vector and an isogenically matched chimeric vector has Ad3 tropism (Ad5/3). Various parameters of virus infection were compared between these two vectors including binding, transgene expression, E1a transcription, *de novo* virus production and oncolysis. At each step of the productive virus replication cycle the chimeric Ad5/3 virus was more efficient compared to its Ad5 counterpart. These data demonstrate the ability to utilize alternative Ad serotype receptors to improve transduction and subsequent oncolytic replication, which is particularly relevant in gene therapy applications for tumors that are inefficiently infected with Ad5.

Introduction

Conditionally replicative adenovirus (CRAd) vectors have emerged as novel therapeutic agents for a variety of neoplastic diseases, which has led to their rapid translation into human clinical trials (1, 3, 6). From a conceptual standpoint, a CRAd agent's anti-tumor effect is measured by its capacity to productively replicate in target tumor cells and ultimately induce oncolysis. This process involves (i) efficient target cell infection, (ii) tumor-specific replication, and (iii) effective dispersion of the viral progeny. The degree that each of these steps are optimized determines the efficacy of the virotherapy. For example, it is generally considered that the binding of the virus to the target cell defines the efficiency of the infection. Also, a greater degree of tumor-specific replication correlates to a higher therapeutic index. Likewise, the capacity of the vector to efficiently disperse enhances the pervasiveness of the infection (10, 17). Deficiencies at these key steps likely explain the inefficacy of CRAds in human clinical applications to date. A consideration of these issues indicates that alternative aspects of CRAd design should be addressed to achieve the full therapeutic potential of these agents.

Whereas CRAd development has primarily focused on improving tumor-specific replication, transduction and dispersion need to also be considered. Many tumor cells have been reported to express relatively low levels of the primary Ad5 receptor, coxsackievirus-adenovirus-receptor (CAR), which essentially renders the tumor cell resistant to Ad5 infection. Clearly, this CAR-deficiency would be likewise consequential in the context of CRAd agents, whereby both the initial infection, as well as the dispersion steps, would be adversely impacted. The recognition that CAR-deficiency is a limiting factor in Ad utility has led to the development of strategies to alter Ad5 tropism

to achieve "CAR-independent" gene delivery. In this regard, CAR-independent Ad5 vectors have been developed that enter cells through a variety of non-native pathways via the EGF, FGF, and folate receptors and integrins (2, 5, 11, 14, 16). These modifications in Ad5 tropism have also resulted in a gain in infection efficiency that directly translates into improved anti-cancer gene therapy effects.

For this study we sought to develop a CAR-independent gene transfer approach to improve the potency of CRAd agents. In this regard, other Ad serotype receptors have been proposed as targets for tumor cell infection. For example, the Ad serotype 3 (Ad3) receptor has been reported as a better target, compared to CAR, in the context of ovian cancer, squamous cell carcinoma of the head and neck, and B cell lymphomas (18, 20). Importantly, the Ad3 receptor has been shown to be distinct from CAR (19). Therefore we evaluated the utility of a chimeric Ad5 vector that has Ad3 tropism (Ad5/3). This vector was generated by substituting the knob domain of the Ad5 fiber protein with that of Ad3. We hypothesized that the chimeric Ad5/3 vector would efficiently infect, replicate and lyse tumor cells that are resistant to Ad5 infection. To test this hypothesis, key steps in the replication cycles were compared between the chimeric Ad5/3 vector and its unmodified Ad5 counterpart. At each step of the virus life cycle – from entry to oncolysis – the chimeric Ad5/3 vector was more efficient. In conclusion, this study suggests that alternative Ad receptors may be useful targets for cancer gene therapy and that the efficacy of CRAd gene therapy may be improved by developing CAR-independent approaches.

Materials and Methods

Cells and viruses. The human SCCHN cell lines, SCC-25, FaDu and SCC-4, were purchased from the American Type Culture Collection, Rockville, MD. FaDu and SCC-25 cells are cultured in Eagle's MEM with Earle's BSS containing 1% L-glutamine, 1 mM non-essential amino acids and 10% fetal calf serum (FCS; Gibco BRL, Gaithersburg, MD). SCC-4 cells are cultured in Ham's F12/DMEM containing 1% L-glutamine, 1 mM sodium pyruvate, 10 mM glucose, 0.2 mg/ml hydrocortisone and 10% FCS. All cell lines are cultured at 37°C in 5% CO_2 atmosphere.

Ad5Luc and Ad5/3Luc were generated at the Division of Human Gene Therapy (UAB). Ad5Luc.RGD, which contains an integrin binding motif (RGD) inserted in the HI loop of the fiber's knob region, has been previously described (4, 11). These three virus are replication defective Ad vectors with the luciferase reporter gene inserted in the E1 region and driven by the CMV promoter. Ad5Luc3 and Ad5/3Luc3 (12) are replication competent vectors with the luciferase reporter gene inserted into the E3 region. Viruses were propagated and plaque titered (8) on 293 cells and purified by double centrifugation on cesium chloride gradients as described elsewhere (7). The physical titers were determined spectrophotometrically by measuring the OD at 260 nm where 1 absorbance unit is equivalent to 1.1×10^{12} vector particles (13).

To metabolically label Ad particles with a radioactive tag, 5 x T175 flask of 293 cells (approximately 90% confluent) were infected with either Ad5Luc3 or Ad5/3Luc3 (20 vp/cell). Twenty-four h later, 0.1 mCi of deoxy-[1', 2', 2, 8-^{3}H]-adenosine 5'-triphosphate (9.25 MBq; Amersham Pharmacia Biotech) was added to each flask. The infection was continued until extensive cytopathic effect was observed (~42 h later).

Infected cells were then harvested and virus was purified as described above. The radioactivity was determined by scintillation counting and physical titer was determined spectrophometerically. The specific activity of Ad5Luc3 (6.4 x 10^{11} vp/ml) and Ad5/3Luc3 (4.2 x 10^{11} vp/ml) was 6.5 x 10^{-5} cpm/vp and 7.9 x 10^{-5} cpm/vp, respectively, using an estimated 60% counting efficiency for tritium.

Binding assay. The [^{3}H]-labeled viruses (above) were used for the binding assays. SCCHN cells were incubated with equivalent amounts of virus (6.5 - 7.9 x 10^4 cpm per 10^5 cells) for 2 h at 4°C. Unbound virus was removed by washing the cell monolayers 2 times with cold media. The cells and [^{3}H]-labeled viruses were then harvested by trypsonization. Radioactivity was measured by scintillation counting to determine the percentage of bound virus (bound ^{3}H counts divided by total input ^{3}H counts times 100).

Reporter gene expression. To quantify adenovirus transgene expression, 10^5 SCCHN cells were plated into 4 replicate wells of 12-well plates in the presence of 2 ml of culture media and were allowed to adhere overnight. The cells were then infected with either Ad5Luc or Ad5/3Luc (50 vp/cell) in 300 µl/well of growth media containing 2% FBS for 2 h at room temperature. The cells were then washed 2 times with growth media and then incubated with fresh growth media at 37°C in 5% CO_2. Twenty-four h after infection, the cells were rinsed with PBS and assayed for luciferase expression. For all luciferase enzyme assays, the cells were lysed in 200 µl of Promega (Madison, WI) lysis buffer. Ten µl of each sample was subsequently mixed with 50 µl of Promega luciferase assay reagent according to the manufacturer's instructions and duplicate determinations of triplicate samples were assayed in a Berthold luminometer.

RT-PCR analysis of E1a transcription. SCCHN cells were infected with Ad5Luc3 or Ad5/3Luc3 (10 vp/cell) as described above. At 0, 3, 6, 12 and 24 h post infection, total RNA was isolated using the RNeasy Mini kit (Qiagen). Prior to RT-PCR, all RNA samples were treated with DNase I for 15 min, 37°C. DNase I was then inactivated by heating at 72°C for 15. All subsequent reverse transcription and polymerase chain reaction (RT-PCR) assays were performed using the OneStep RT-PCR kit (Qiagen). For the non-quantitative analyses, E1a primers (5'-ACG GTT GCA GGT CTT GTC ATT ATC A-3' and 5'-AAG CAA GTC CTC GAT ACA TTC CA-3') and GAPDH primers (5'-TCC CAT CAC CAT CTT CCA-3' and 5'-ACC TTC TAC CAC TAC CCT-3') were used. Since this E1a primer pair flanks an intron it can be used to distinguish between PCR products generated from E1a mRNA templates (351 bps) and E1a DNA templates (478 bps). In all cases, DNase I pretreatment of the total RNA was optimized to eliminate contaminating E1a DNA. The RT-PCR thermal cycles were as follows: 50°C (30 min) and 94°C (15 min) for 1 cycle; 94°C (1 min), 55°C (0.5 min) and 72°C (1 min) for 25 cycles; 72°C (10 min) for 1 cycle.

Quantitative RT-PCR analysis of E1a transcription. For quantitative RT-PCR, total RNA was isolated and treated with DNase I as described above. The forward primer (5'-AAC CAG TTG CCG TGA GAG TTG-3'), reverse primer (5'-CTC GTT AAG CAA GTC CTC GAT ACA T'3') and 6-FAM labeled probe (5'-CAC AGC CTG GCG ACG CCC A-TAM RA) were used to amplify the E1A gene and the forward primer (5'-GGT TTA CAT GTT CCA ATA TGA TTC CA-3'), reverse primer (5'-ATG GGA TTT CCA TTG ATG ACA AG-3'), and 6-FAM labeled probe (5'-CGT TCT CAG CCT TGA CGG TGC CA-3') was used to amplify glyceraldehyde-3-phosphate dehydrogenase gene

(GAPDH). All primers and probes were designed using the Primer Express 1.0 software (Perkin Elmer, Foster City, CA) following the recommendations of the manufacturer. RT-PCR was then performed in a mixture with a final volume of 9 µl per reaction containing 1X TaqMan EZ buffer, 3 mM of Mangnese acetate, 300 µM of dATP, dCTP, dGTP, 600 µM of dUTP, 100 nM of forward and reverse primer, 100 nM probe, 0.1 U/µl of r*Tth* DNA Polymerase, 0.01U/µl of AmpErase UNG, 0.025% BSA and RNase-free water. For each experiment a known amount E1A template RNA (10^8, 10^6, 10^4 and 10^2 copies/µl) was used as a standard cure to quantify the E1A copy numbers of the experimental samples. A known amount of total RNA (25, 5, 1 and 0.1 ng/µl) was used to generate a standard cure to quantify total RNA based on GAPDH copies. To generate a standard curve for determining E1a copy numbers, known amounts of E1A cRNA were used following *in vitro* transcription (AMBION MAXIscript In Vitro Transcription Kit, Ambion, Austin, TX). A sample of the total RNA (1.0 µl) from each experimental sample or the standard curve samples (total RNA and E1a cRNA) was added to the 9 µl of RT-PCR mixture in each reaction capillary. Three no template controls received 10 µl of reaction mixture with 1 µl of water. All capillaries were then sealed, mixed, and subjected to TaqMan Real-Time PCR in a LightCycler™System (Roche Molecular Biochemicals, Indianapolis, Indiana). Thermal cycling conditions were as follows: 2 minutes at 50 °C, 30 minutes at 60 °C, 5 minutes at 95 °C, and 40 cycles of 20 seconds at 94 °C and 1 minute at 62 °C. Data was analyzed with LightCycler software.

Quantification of virus yield. SCCHN cells were infected with either Ad5Luc3 or Ad5/3Luc3 at 10 vp/cell. After the 2 h adsorption period at 4°C, the cell monolayers were washed 2 times with fresh growth media and then cells were incubated in 1 ml of

fresh growth media at 37°C in a CO_2 incubator. Forty-eight h post infection, the cells were harvested by scraping into the growth media. The cells were subjected to 4 freeze-thaw cycles and then centrifuged at 10,000 x g for 15 min at room temp. The amounts of virus present in the resulting supernatants were then analyzed by a plaque assays on 293 cells as described previously (8).

Cytotoxicity assays. SCCHN cells were either mock-infected or infected with Ad5Luc3 or Ad5/3Luc3 at different. Twelve h later, the cells were trypsinized, counted and plated (5000 cells/well) into 12-well culture plates in 4 replicates. Five days post infection the number of viable cells was determined using the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay kit (Promega). This colorimetric assay measures the conversion of a tetrazolium compound to formazan by viable cells. On the assay day, media from each well was aspirated, replaced with 400 µl of fresh growth media containing 80 µl of the cell proliferation assay reagent, and incubated at 37°C for approximately 1 h. Four h prior to the start of the assay, known numbers of uninfected cells were plated in triplicate wells to generate a standard curve for determining the number of viable cells in the experimental samples. These cells received the cell proliferation assay reagent at the same time as the experimental cells. A 100 ml aliquot of the media from each well was transferred to a 96-well microtiter plate and the absorbance at 490 nm was measured in a plate reader (Molecular Devices, Menlo Park, CA). Data collected by the plate reader was analyzed by the SOFTmax software package (Emax Molecular Devices, Menlo, CA) and the number of viable cells was determined.

Results

Gene therapy for several tumor types has been thwarted due in part by their relative resistance to Ad5 infection. We have previously shown that Ad5 resistance can be overcome by rerouting entry of the vector to non-native receptors such as EGFR, FGF, folate, CD40 and integrins (2, 9, 21). In this study we evaluated the use of the Ad3 receptor, which has been shown previously to have a binding specificity distinct from that of the Ad5 receptor (12). Chimeric Ad5 vectors containing the knob region of Ad3 were compared to Ad5 vectors at several of the key steps in a productive Ad infection including binding, E1a transcription, gene expression, *de novo* virus production, and oncolysis.

Chimeric Ad5/3 virus has higher binding activity. *In vitro* binding studies were performed to assess the binding activities of the Ad5 and chimeric Ad5/3 viruses. The genomes of each virus were metabolically labeled with tritiated dATP and then the viruses were purified by standard methods (7). Equivalent amounts of each virus were then incubated with SCCHN cells for 2 hours at 4°C. Under these conditions the virus binds to the cells but does not enter (15). The amounts of bound and unbound virus were then measured by scintilation counting. For each cell line tested, the chimeric Ad5/3 virus had higher binding activity (Figure 1). These data suggest that (i) the number of Ad3 receptors is higher on SCCHN cells compared to CAR and/or (ii) that the Ad3 receptor-Ad3 knob interaction has a higher affinity compared to the CAR-Ad5 knob interaction.

A higher level of E1a transcription is detected with the chimeric Ad5/3 virus. Next, we compared early gene transcription (i.e., E1a) from either the Ad5 and Ad5/3

vectors at various time points after infection (0, 3, 6, 12 and 24 h). Total RNA was extracted and analyzed by RT-PCR using E1a-specific primer pairs (Figure 2A). E1a mRNA was detectable12 hour time point after infection with the Ad5 virus (Figure 3A, top panel) and was detectable 6 hour point after infection with the chimeric Ad5/3 virus (Figure 3A, bottom panel), indicating that infection by the chimeric Ad5/3 virus resulted in more E1a transcription products at early time points. Real time TaqMan RT-PCR was also performed to quantitatively determine the amounts of E1a mRNA at these time points (Figure 3B). At each time point tested, higher copy numbers of E1a mRNA were detected following infection by the chimeric Ad5/3 vector. On average, approximately 12-fold more E1a mRNA was transcribed over the first 24 h post infection with the chimeric Ad5/3 vector. Although the absolute amounts of E1a mRNA differed between the two viruses, the rate of increase of E1a mRNA accumulation was same for both the Ad5 and chimeric Ad5/3 vectors, suggesting that there was not a difference is the kinetics of E1a transcription between the two vectors.

Higher reporter gene activity after infection with the chimeric Ad5/3 virus. To determine if reporter gene expression was different following infection, SCCHN cells were infected with the Ad5 and chimeric Ad5/3 virus. In addition, we have previously shown that another genetically modified vector, which contains an RGD motif inserted into the HI loop region of the Ad5 knob, more efficiently infects SCCHN cells (9). Therefore, this vector was also compared in this analysis. SCCHN cells were infected with equal amounts of each virus (50, 500, or 5000 vp/cell) and transgene activity (i.e., luciferase) was measured approximately 36 h later (Figure 3). Consistent with our previous studies, reporter gene activity following infection by Ad5Luc.RGD virus was

higher in each of the SCCHN cell lines tested. Strikingly, reporter gene activity was even greater following infection by the chimeric Ad5/3 virus. These data demonstrate that at least two non-native receptor pathways can be exploited to augment the efficiency of Ad infection.

***De novo* virus production is higher with the chimeric Ad5/3 virus.** The difference in E1a transcription (shown in Figure 2) between the two viruses predicts more efficient production of progeny virus by the chimeric Ad5/3 vector. To test this possible outcome, SCCHN cells were infected with equal amounts (10 vp/cell) of either the Ad5 or chimeric Ad5/3 viruses. Forty-eight h after infection, the amount of *de novo* virus produced was determined by plaque assays (functional viral particles) on 293 cells and spectrophotometric (total viral particles) analyses (Figure 4A). Depending on the SCCHN cell initially infected, between 200- to 250-fold more virus was produced following infection by the chimeric Ad5/3 virus. One possible explanation for this result was that 289 cells have a disproportionate amount of the Ad3 receptor relative to the Ad5 receptor, which would have artificially skewed these results in favor of the chimeric Ad5/3 vector. To test this possibility, 293 cells were infected with equal numbers of vector particles (10 vp per cell) and approximately 48 h later the resulting numbers of PFU and vp were determined (Figure 4B). The 293 cells produced approximately equal amounts of functional and total particles of each virus, validating the results with the SCCHN cells (Figure 4A). The VP-to-PFU ratio, which is an index of the quality of the virus preparation, was 25 and 21 for Ad5Luc3 and Ad5/3Luc3, respectively, indicating that there was no difference in the capacity of the 293 cells to produce either virus.

Oncolysis is more efficient with the chimeric Ad5/3 virus. We next compared oncolytic replication between the Ad5 and chimeric Ad5/3 vectors. SCCHN cells were infected with each virus and approximately 5 days later the monolayers were stained with crystal violet to assess oncolysis (Figure 5, Panel A). For each cell line tested more oncolysis was observed following infection by the chimeric Ad5/3 virus. To quantitatively evaluate oncolysis by the two viruses, SCCHN cells were infected with escalating amounts of each virus (0 to 100 vp per cell) and then 3 days later cell viability assays were performed (Figure 5, Panel B). In each instance, substantially less of the chimeric Ad5/3 virus was needed to achieve efficient cell oncolysis. Collectively, these data demonstrate that in tumor cells that are relatively refractory to Ad5 infection, the chimeric Ad5/3 virus is capable highly efficient oncolytic replication.

Discussion

The modification of vector tropism has been an important issue during Ad development. Both untargeting of the vector from the native receptor and retargeting of the vector to heterologous receptors have been critical advancements for improving the utility of Ad-based gene therapy. These developments have been needed to achieve targeting of specific cells and tissues, to reduce non-target cell infection and to increase the efficiency of infection. Immunological, chemical and genetic modifications have been applied to alter vector tropism. In addition, the native tropism can be substituted by swapping the knob regions of the fiber proteins of different Ad serotypes. The resulting chimeric vectors have been shown to have different neutralization profiles and efficiencies for transducing different cell types. In general however, these studies typically use replication-incompetent Ad vectors with quantifiable reporter genes (e.g. luciferase, GFP) for determining transduction efficiency. In this study we hypothesized that Ad vector tropism can be modified to also produced more effective oncolytic Ad vectors. A model system using isogenically matched, replication-competent Ad vectors with either Ad5 or Ad3 tropism were used to test this hypothesis. Our study showed that each step of the virus replication cycle was enhanced by routing the vector to the serotype 3 receptor. A modest improvement in vector binding to the target tumor cell greatly amplified the oncolytic activity of the chimeric Ad5/3 vector. For example, a 3- to 6-fold increase in binding translated into 2- to 2.5-log higher oncolytic activity. Although we cannot rule out the possibility that the Ad5 and Ad5/3 vector had different replication kinetics, it seems unlikely since the rate of early gene transcription was essentially the same for both vectors (see Figure 2B). A more likely explanation is that since we used

relatively low MOIs for the infection conditions, multiple rounds of infection occurred that amplified the differences in the binding kinetics between the two vectors. It is also possible that our binding data, which was generated under one vector-to-cell ratio experimental condition, does not fully represent the kinetics of binding during a productive replication where the ratio of vector-to-cell is constantly changing over time due to the accumulation of virus in the media. Importantly, this study underscores the importance of tropism-modification to improve the efficacy of Ad-based gene therapy. It shows the possible utility of using alternate serotype tropisms for tumors that are refractory to Ad5. As well, it reminds us of the need for the characterization of other serotype receptors and the possible role they may have in tumor development and therapies.

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Figure legends

Figure 1. Binding activities of Ad5 and chimeric Ad5/3 viruses. Known amounts of [H^3]-labeled Ad5Luc3 and Ad5/3Luc3 virus particles were incubated with monolayers of SCCHN cells for 2 hours at 4°C. Unbound virus was then removed by washing and the amount of bound virus was determined by scintillation counting. The graph shows an average of replicate samples (n = 3) from 2 independent experiments.

Figure 2. E1a transcription following infection by Ad5 and chimeric Ad5/3 viruses. SCCHN cells (FaDu) were infected with an equal amounts (20 VP per cell) of either Ad5Luc3 or Ad5/3Luc3 and then at different time points after infection (0, 3, 6, 12 and 24 h) total RNA was isolated and subjected to RT-PCR analyses. **A.** Time course of E1a transcription. Triplicate wells of FaDu cells were infected with Ad5Luc3 (top two panels) or Ad5/3Luc3 (bottom two panels) and then analyzed with RT-PCR using E1a and GAPDH primer pairs. **B.** Quantitative RT-PCR analysis of E1a transcription. mRNA from the same samples shown in Panel A were subjected to quantitative RT-PCR analysis. A standard curve was generated from known amounts of E1a mRNA to calculate the number of E1a mRNA copies in the experimental samples. RT-PCR analysis of GAPDH mRNA was used to normalize differences in total RNA in experimental samples. The graph shows the average (n = 3) of normalized E1a mRNA copy numbers at different time points after infection.

Figure 3. Reporter gene expression following infection by Ad5 and chimeric Ad5/3 viruses. SCCHN cells were infected with equal amounts (0, 50, 500 or 5000 VP

per cell) of Ad5Luc1 and Ad5/3Luc1. Approximately 30 hours later, luciferase activity was measured. The graph represents the average of replicate samples (n = 4) with error bars indicating the standard deviation between samples. The average background luciferase activity was subtracted from all experimental values.

Figure 4. Progeny virus production after infection with Ad5 or chimeric Ad5/3 virus. A. SCCHN cells were infected with either Ad5Luc3 or Ad5/3Luc3 (10 VP per cell). Forty eight h later the media and cell fractions were harvested, pooled and analyzed for *de novo* Ad production. Functional virus was measured by plaque assays on 293 cells and total virus was measured spectrophotometrically. The graph represents the average of replicate samples (n = 4) with error bars indicating the standard deviation between samples. **B.** Exactly as in Panel A except that 293 cells were infected with the viruses. The graph shows the results amounts of functional virus (PFU) and total virus (VP) produced.

Figure 5. Comparison of oncolytic replication between Ad5 and chimeric Ad5/3 viruses. A. SCCHN cells were mock-infected or infected with either Ad5Luc3 or Ad5/3Luc3 (10 VP per cell). Ninety-six h later the monolayers were stained with crystal violet. **B.** Replicate wells of SCCHN cells were mock-infected or infected with different amounts of either Ad5Luc3 or Ad5/3Luc3 (0.01, 0.1, 1.0, 10, or 100 VP per cell). Ninety-six h later the numbers of viable cells were measured by cell proliferation assays. The graph represents the average of replicate samples (n = 4) with error bars indicating the standard deviation between samples.

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Advances in Brief

Heat Shock and Heat Shock Protein 70i Enhance the Oncolytic Effect of Replicative Adenovirus[1]

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Abstract

Replication-competent viruses are currently being evaluated for their cancer cell-killing properties. These vectors are designed to induce tumor regression after selective viral propagation within the tumor. However, replication-competent viruses have not resulted heretofore in complete tumor eradication in the clinical setting. Recently, heat shock has been reported to partially alleviate replication restriction on an avian adenovirus (Ad) in a human lung cancer cell line. Therefore, we hypothesized that heat shock and overexpression of heat shock protein (hsp) would support the oncolytic effect of a replication-competent human Ad. To this end, we tested the oncolytic and burst kinetics of a replication-competent Ad after exposure to heat shock or to inducible hsp 70 overexpression by a replication-deficient Ad (Adhsp 70i). Heat-shock resulted in augmentation of Ad burst and oncolysis while decreasing total intracellular Ad DNA. Overexpression of hsp 70i also enhanced Ad-mediated oncolysis but did not decrease intracellular Ad DNA levels. We conclude that heat shock and Adhsp 70i enhance the Ad cell-killing potential via distinct mechanisms. A potential therapeutic implication would be the use of local hyperthermia to augment oncolysis by increasing the burst of replication-competent Ad. The role of hsp in Ad-mediated oncolysis should be additionally explored.

Introduction

Because malignant tumors are of a highly complex nature, a cure will probably result only from complete eradication of all of the tumor cells. Current modalities for cancer therapy are not selective and may have severe adverse effects. Therefore, cancer gene therapy has emerged as an alternative and promising new modality after the recent advances in understanding cancer biology. Despite the initial encouraging preclinical studies using replication-incompetent viral vectors, these agents failed to result in significant benefit in the clinical setting. A natural evolution of this course was the introduction of RCVs[3] for cancer therapy (1), where selective viral self-perpetuation is suggested to eradicate or decrease tumor mass and augment the host immune response to tumor antigens. Despite the use of other vectors, human Ads are the leading agents for cancer gene therapy. Whereas the life cycle of Ad has not been completely elucidated, it is clear that it requires redirection of the host cellular biochemical machinery by viral gene products. Viral replication is dependent on the host cell for genome replication, protein synthesis, and virion assembly. In this regard, hsps may play an important role in the Ad life cycle (2). It has been demonstrated for the avian Ad CELO that induction of hsp 40 and 70 allowed production of viral proteins and virions. Importantly, the restriction on a mutant CELO Ad replication was alleviated by both heat shock and hsp overexpression. Previous supportive data regarding the role of hsp in Ad infection also include correlation of hsp levels with permissiveness of human cells to Ad infection (3) and selective hsp 70 mRNA transport occurring late in Ad infection (4).

Consequently, we hypothesized that heat shock and hsp overexpression would alter Ad life cycle in cancer cells to enhance oncolysis.

To test our hypothesis, we heat-shocked lung cancer cell lines after Ad infection and demonstrated augmentation of Ad burst and oncolysis. Next, we overexpressed the inducible hsp 70 with a replication-incompetent Ad vector and have shown that it also enhances Ad-mediated oncolysis. However, Ad burst patterns differ after cellular exposure to heat shock or hsp 70i overexpression.

Materials and Methods

Cell Lines and Viruses. The lung cancer cell lines A549 and H460 were obtained from the American Type Culture Collection (Manassas, VA), and H157 was a gift of Koicihi Takayama (Princeton University, Princeton, NJ). An E3-deleted, replication-competent human Ad5 expressing luciferase (Ad5luc3) was used in this study as a replication-competent equivalent of wild-type human Ad5. An E1-deleted, replication-incompetent Ad5 expressing luciferase (Ad5luc1) was used as a control virus. Ad338 is a replication-competent, E1B-55,000-deleted Ad5, kindly provided by Tom Shenk (5). Adhsp70i is an E1-deleted Ad expressing the inducible hsp 70 under the transcriptional control of the human CMV enhancer/promoter and was obtained from Ruben Mestril (University of California, San Diego, La Jolla, CA) (6).

Infections, Heat Shock, and Cell Viability Assays. A549, H460, and H157 cells were grown in a Ham's F12K medium with 2 mM L-glutamine, supplemented by 10% fetal bovine serum at 37 C°. For the heat shock experiments, 2×10^5 cells/well were plated in 12-well plates and on the next day were infected with either Ad5luc3 or Ad5luc1 in triplicates with MOI of 0, 0.01, 0.1, or 1 plaque-forming unit/cell. Serum-free infection medium was removed after 1 h and replaced with 2 ml of 5% fetal bovine serum growth medium. The medium was not replaced thereafter during the experiment and was sampled daily for future determination of Ad5 E1a gene copy numbers. For the heat shock experiments, plates were incubated in a Forma Scientific incubator (Marijetta, OH) at 37 C° and heat shocked daily at 42.5 C° for various periods in a distinct designated incubator. Temperature was validated with two different thermometers.

Plates were simultaneously stained with crystal violet once an advanced CPE was identified for either of the groups. For the experiments with Adhsp 70i, A549 cells were grown as before. A549 cells were infected with Adhsp 70i at an MOI of 10 for 1 h in a serum-free medium. Ad5luc1 served as a control replication-incompetent Ad. After 30 h, we infected with the replication-competent Ad5luc3 at an MOI of 5 under the same conditions as above. Medium was sampled daily for E1a gene copy numbers, and all of the plates were stained simultaneously with crystal violet once an advanced CPE was identified for either of the groups. Quantitative cell killing evaluation of heat

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[3] The abbreviations used are: RCV, replication-competent virus; Ad, adenovirus; hsp, heat shock protein; MOI, multiplicity of infection; CPE, cytopathic effect; HPV, human papillomavirus; Tag, T antigen.

shocked cells was performed with the MTS assay using the CellTiter 96 AQ Cell Proliferation assay (Promega, WI), modified for 24-well plates. Cells were infected at an MOI of 1 and evaluated at different time points as depicted in Fig. 1*B*. MTS reagent (80 μl) was added to each well before determination of OD by 490 nm absorbance of formazan measured directly from the 24-well plates.

TaqMan PCR Assay. E1a copy numbers were determined for each medium sample obtained in triplicates as of the first day after infection. Genomic DNA was isolated and cleaned using a Qiagen Tissue kit (Qiagen, Santa Clara, CA) according to the instructions of the manufacturer. Concentration of purified DNA was determined by A260. The design of TaqMan primers and probe was as follows: the forward primer, reverse primer, and 6-FAM labeled probe to amplify the *E1a* gene were designed by the Primer Express 1.0 software (Perkin-Elmer, Foster City, CA) and are available on request.

With optimized concentration of primers and probe, the components of real time PCR mixture were designed to result in a master mix with a final volume of 10 μl/reaction containing 1X Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 100 nM forward primer, 100 nM reverse primer, 1 nM probe, and 0.025% BSA. For the assay, 1 μl of extracted DNA sample was added to 10 μl of PCR mixture in each reaction capillary. A no-template control received 10 μl of reaction mixture with 1 μl of water. All of the capillaries were then sealed and centrifuged using LC Carousel Centrifuge (Roche Molecular Biochemicals, Indianapolis, IN) to facilitate mixing. All of the PCR was carried out using a LightCycler System (Roche Molecular Biochemicals). The thermal cycling conditions were 10 min at 95°C, 40 cycles of 15 s at 95°C, and 1 min at 60°C.

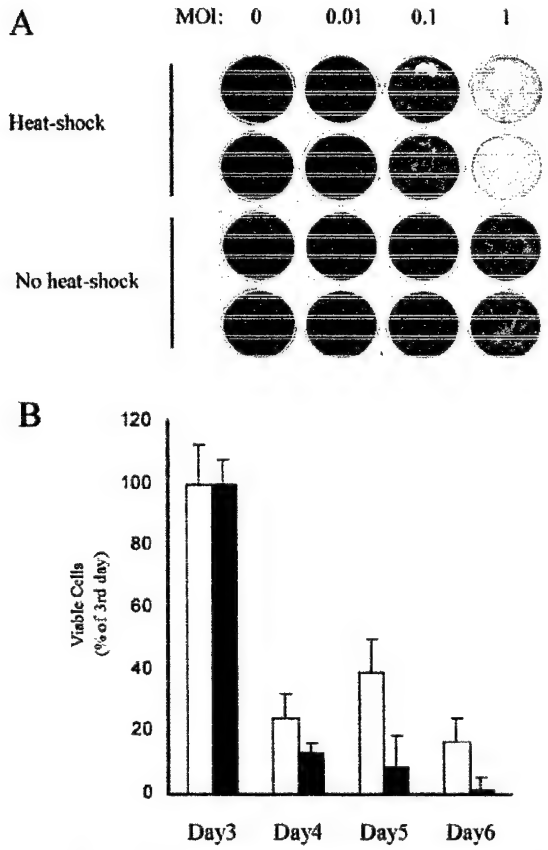

Fig. 1. Heat shock enhances the oncolytic effect of a replicating Ad. *A*, A549 cells were mock-infected or infected with Ad5luc3 with MOIs of 0.01, 0.1, or 1, and then either exposed to a daily 1 h heat shock (*top panel*) or not (*bottom panel*). Cells were stained with crystal violet after observation of an advanced CPE. *B*, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay depicting the kinetics of Ad-mediated cell killing as affected by heat shock. A549 cells were infected by Ad5luc3 at an MOI of 1 and subject either to daily 1-h heat shock (■) or not (□). Cell viability was determined by optic density (OD) reading of individual wells and is an average of 6 wells; *bars*, ±SD. Time interval between day 4 and 5 was 14 h.

Statistical Analysis. Data were initially tested for normality by the Shapiro-Wilk test. All of the abnormal tests were additionally tested for significance by the Wilcoxon-scores test. Results are expressed as a mean of at least three samples for E1a copy number assay or six samples for the MTS assay. Results were considered statistically significant for a $P < 0.05$.

Results

Heat Shock Augments the Oncolytic Effect of Replicative Ad. To evaluate the hypothesis that the oncolytic potency of replicating Ad is enhanced by heat shock, we first examined the effect of hyperthermia on Ad5luc3-mediated oncolysis in A549 cells. Heat shock induces apoptotic cancer cell killing at temperatures ~42°C, whereas at higher temperatures cell death is necrotic (7). Another important result of heat shock is the induction of a variety of hsp (8). Whereas the maximal rate of hsp synthesis occurs 3–5 h after heat shock, hsp levels may remain elevated for days after hyperthermic exposure. To evaluate the effect of heat shock on Ad-mediated oncolysis, we heat shocked A549 cells as of the first day after infection for either 1 or 2 h daily. For these studies, Ad5luc3 was selected, because it is an E3-deleted replicating virus expressing luciferase. Therefore, we eliminated the potential lytic role of the Ad death protein and normalized for transgene expression with the control replication-incompetent virus, Ad5luc1. Consequently, the variable determining the outcome of infection was Ad5 life cycle within the tumor cells. To normalize for heat-induced toxicity, in each 12-well plate a triplicate was mock-infected, thereby subject only to the effect of the variable heat shock periods. We selected 42.5°C as an optimal temperature for heat shock induction, because Ad5 is partially inactivated at 43–44°C, and because temperature below 42°C may yield less than optimal hsp induction (8). Longer periods of heat exposure were toxic to the cells, whereas repetitive exposures up to 2–4 h/day in all, were tolerable. To carefully characterize the heat shock effect on viral burst and oncolysis, we used low MOIs, from 0.01 to 1 viral plaque-forming unit/cell.

The replication-incompetent Ad5luc1 did not induce any CPE for the MOIs tested, with or without heat shock, for as long as 14 days after infection. However, for the replicating Ad5luc3, CPE was evident as of 4–5 days after infection. The earliest CPE was documented for cells infected with an MOI of 1 and exposed to daily heat shock (Fig. 1*A*). Cells infected with same MOI that were not heat shocked displayed an equivalent degree of CPE at least 24 h later, whereas cells that were heat shocked 2 h daily had a higher degree of CPE than cells heat shocked for 1 h daily. Of note, the degree of CPE obtained for cells heat shocked for 2 h daily after infection with 0.01 or 0.1 MOI was equivalent at that time point to that observed for non-heat shocked cells infected with an MOI 1–2 order of magnitude higher. These findings are in agreement with both the characteristics of time-dependent heat shock induced apoptosis (9) and time-dependent hsp 70 induction (10), and indicate that the length of the heat shock affects Ad-mediated oncolysis. Because mock-infected cells exposed to identical heat shock periods grew as well as the nonheated cells (Fig. 1*A*, *left lanes*), the conditions of heat shock we used did not induce cell toxicity independently but rather contributed synergistically to Ad5-mediated oncolysis. Thus, controlled hyperthermia supports Ad5-mediated oncolysis.

To analyze quantitatively the effect of heat shock on the *in vitro* Ad oncolysis, an MTS cell viability assay was performed (Fig. 1*B*). The infection protocol for the MTS assay was identical to the crystal violet assay. The cell killing pattern induced by heat shock suggests different kinetics for the heat shocked groups where cell viability rapidly deteriorates, whereas the rate of the non-heated cell killing is moderate. In comparison to the heat shocked group, the nonheated cells had a late and relatively slower rate of lysis.

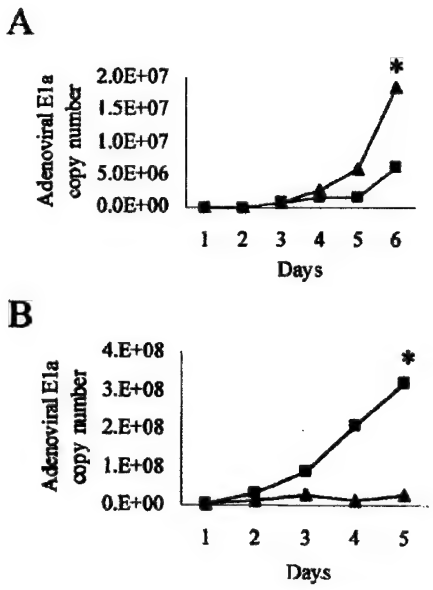

Fig. 2. Heat shock induces Ad burst from cancer cells. *A*, kinetics of Ad burst in A549 as evaluated by quantitative TaqMan PCR. E1a gene copy number was measured in medium samples taken in triplicates as of the first day after infection with Ad5luc3 at an MOI of 1. Plates were either heat shocked at 42.5°C (▲) or not (■) for 1 h daily. $*P < 0.05$ for significance of heat shock as a determinant of E1a gene copies in the medium 6 days after infection with the replicative Ad5luc3. *B*, intracellular Ad DNA levels as reflected by Ad E1a gene copy number. Three days after infection exactly as in *A* total DNA was extracted in triplicates from either heat-shocked cells (▲) or non-heat-shocked (■). $* P < 0.05$ for significance of heat shock as a determinant of intracellular E1a levels 5 days after infection.

These experiments confirmed the enhanced effect of controlled heat shock on Ad5 oncolysis and were also documented for two other lung cancer cell lines, H460 and H157. As well, other replication-competent recombinant Ad5 viruses, including the attenuated E1B-55,000-deleted Ad 338, had a higher oncolytic potency in combination with heat shock (data not shown). Thus, heat shock-enhanced Ad oncolysis may be a general feature of replication-competent Ad.

Heat Shock-enhanced Adenoviral Oncolysis Is Mediated by Facilitated Viral Burst. To investigate the mechanism of heat shock-enhanced Ad5 oncolysis, we evaluated the patterns of Ad5 release from A549 cells. Ad viruses lyse cells after their replication and release from the cell. Therefore, we reasoned that determination of the kinetics of Ad5 release from the cells would elucidate the mechanism of heat shock-enhanced oncolysis. To this end, we quantitatively evaluated Ad DNA in the medium and cellular fractions by determining Ad5 E1a gene copy numbers with the TaqMan assay, as used previously to quantitate Ad DNA (11). Analysis of Ad DNA in the medium clearly indicates that heat shock results in earlier and higher viral release to the medium (Fig. 2*A*). The heat-induced Ad burst is in accordance with the augmented cell killing effect of heat shock on Ad oncolysis, suggesting that it represents the mechanism of heat-enhanced Ad oncolysis. Because either augmented viral replication or earlier cell death may be the primary event resulting in earlier Ad burst, we additionally analyzed the number of intracellular Ad E1a gene copies in various time points after infection as an index of Ad replication (Fig. 2*B*). The nonheated cells produced continuously rising numbers of Ad DNA copies, indicating ongoing Ad replication and infection. In contrast, in the heat shocked cells, the total Ad5 gene copy number leveled off 2–3 days after infection.

Therefore, augmented Ad replication is not the primary mechanism of heat-enhanced oncolysis. Rather, primary heat-induced Ad burst may account for the enhanced cell killing, the rising concentration of Ad DNA in the medium, and for the constantly low intracellular Ad DNA levels. Heat shock may result in premature death of infected cells, rendering Ad progeny available for more infections. Alternatively, heat shock may affect directly the life cycle of Ad. Whereas these two scenarios need additional studies, our data clearly show that for the *in vitro* cell killing assays we used, hyperthermia alone is insufficient to achieve significant cancer cell killing, whereas it dramatically alters the natural course of cellular infection with a replicating Ad. Of note, we also demonstrated induction of Ad5 burst by irradiation (data not shown), but this phenomenon requires additional studies. Taken together, our data indicate that heat shock efficiently induces *in vitro* a shift of Ad5 from the cellular component to the medium, representing earlier Ad burst and cell death.

Adhsp 70i Supports Adenoviral-mediated Oncolysis. After the determination of the synergistic effect of heat shock on Ad5 burst and oncolysis, we turned to examine the direct effect of hsp 70i, the major cellular stress protein, on Ad-mediated cell killing. We hypothesized that hsp 70i overexpression by the replication-deficient Ad5hsp 70i vector would augment the oncolytic effect of a replicating Ad5. To this end, we preinfected A549 cells at an MOI of 10 with the replication-incompetent viruses, either Adhsp 70i or the control Ad5luc1. Later (30 h), cells were infected with the replication-competent Ad5luc3 at an MOI of 5.

A simultaneous coinfection of a replicating virus and either one of these nonreplicating viruses resulted in rapid oncolysis at both groups, probably caused by E1 *trans*-complementation of the nonreplicating viruses. Therefore, to decrease the *trans*-complementation and delineate the isolated effect of hsp 70i overexpression on Ad5 replication and oncolysis, the time interval from infection with the replication-deficient Ad was essential. As early as 36 h after infection with Ad5luc3, we could notice typical CPE for the Adhsp 70i preinfected group. At this stage, no CPE was apparent for the cells infected with either the replicating virus alone or in combination with Ad5luc1 (Fig. 3*A*). Therefore, hsp 70i overexpression by Adhsp 70i is synergistic to the cell killing effect of replicative Ad. Next, we sought to evaluate the effect of Adhsp 70i on the kinetics of Ad5luc3. Infection with Adhsp 70i or Ad5luc1 was performed exactly as for the cell killing assay. We simultaneously sampled daily both the medium, and from a different set of plates, the cellular fraction, for E1a copy number measurement with the quantitative TaqMan PCR. When compared with Ad5luc3 in combination with the control Ad5luc1, Adhsp 70i induced an earlier and higher Ad5luc3 burst rate, as determined by the quantitative determination of Ad5 gene copies in the medium (Fig. 3*B*). However, this difference did not reach statistical significance. When evaluating the cellular fraction of Ad5luc3 E1a gene copies in cells preinfected with Adhsp 70i, we found that viral DNA was more abundant in comparison to the cells preinfected with Ad5luc1 (Fig. 3*C*). Although this difference was also not statistically significant, it is clearly distinct from the inhibition of cellular Ad DNA accumulation induced by heat shock. These data may indicate that Adhsp 70i confers a selective advantage for Ad5-mediated cell killing, but unlike heat shock, not via an isolated effect on Ad5 burst.

Discussion

Cancer gene therapy is currently limited by the inadequacy of vectors to completely eliminate the malignant clone. Therefore, RCVs have been proposed to lyse cells and propagate throughout the tumor mass. However, clinical trials with local injection of RCV for head and neck cancer have shown that whereas an acceptable safety level was achieved, oncolytic efficacy clearly requires improved potency (12). In this regard, we have investigated the hypothesis that hyperthermia and hsp 70i overexpression would support Ad5-mediated

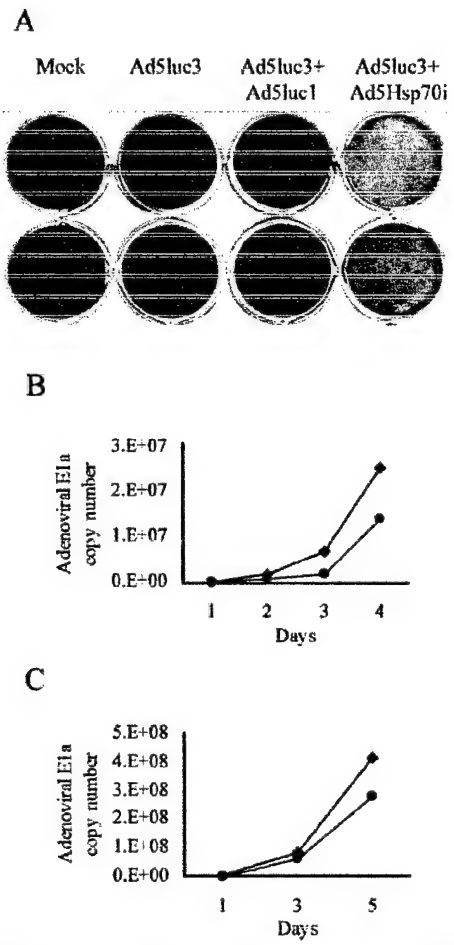

Fig. 3. Hsp 70 overexpression by Adhsp 70i augments Ad burst and cell killing. *A*, the human lung adenocarcinoma cell line A549 was infected at an MOI of 10 with the replication-incompetent vectors, either the control virus Ad5luc1 (*Lane 2 from right*), or Adhsp 70i (*right lane*). Later (30 h), cells were infected with the replication-competent Ad vector Ad5luc3 at an MOI of 5. Cells were stained with crystal violet after observation of advanced CPE. Mock represents mock-infected cells. *B*, kinetics of Ad burst after hsp 70i overexpression. A549 cells were infected exactly as in *A* with Ad5luc1 (●) or Ad5hsp 70i (♦). Later (30 h), all of the cells were infected with the replication-competent Ad5luc3 at an MOI of 5. Medium was sampled daily in triplicates as of the first day after infection with Ad5luc3 for future determination of E1a gene copy number. *C*, analysis of intracellular Ad DNA replication by determination of E1a gene copy number. A549 cells were infected exactly as in *A* with Ad5luc1 (●) or Adhsp 70i (♦), and 30 h later, cells were infected with the replication-competent Ad5luc3 at an MOI of 5. On the indicated time periods after infection with Ad5luc3, cells were harvested for total DNA extraction.

oncolysis. To address this hypothesis, we first evaluated the cell killing effect and the kinetics of Ad5 burst after a daily heat shock. We have shown that a controlled heat shock augments Ad5 burst, thereby increasing its oncolytic potency. Because the combination of heat shock and a replication-deficient Ad5 did not increase cell killing, this effect of heat shock is unique to replication-competent Ad. Finally, we demonstrated that Ad5 burst is supported by Adhsp 70i, albeit via an alternate mechanism that is not restricted to viral burst. These findings are reminiscent of the paradoxical enhancement of viral CPE by the canine distemper virus after induction of the stress response (13). Our findings also agree with clinical data, where isolated hyperthermic therapy may have no significant independent therapeutic effects at the available hyperthermic strategies (14). In this regard, our studies indicate that the combination of heat shock and Ad5 replication increases the *in vitro* potency of Ad-mediated cell killing. The mechanism of heat-enhanced Ad oncolysis stems from alteration of the natural viral life cycle in the cell lines we used. The earlier virus discharge from the cell correlates with cell killing, and both are enhanced by heat shock.

One mechanism to explain this observation may involve the induction of apoptosis by heat. While by itself insufficient to result in significant cell killing in our study, heat shock can efficiently induce apoptosis (7, 15). Importantly, induction of apoptosis by exposure to 42°C in combination with chemotherapy has been reported to be more efficient than by hyperthermia of 44°C (16). We speculate that apoptosis may affect the release of viral progeny after completion of the viral life cycle. This hypothesis is currently being studied. Another attractive mechanism for heat shock-enhanced oncolysis would be the induction of hsp. In this regard, our studies showed that hsp 70i overexpression also resulted in a higher degree of cell killing. However, unlike heat shock, Ad replicated well in the cellular fraction after hsp 70i overexpression. We could not demonstrate a statistically significant increase in Ad replication after hsp 70i expression, but DNA levels of the replication-competent Ad were higher both at the cellular level and in the medium. Because cellular Ad DNA levels were time-dependent after infection, it seems that isolated viral burst is not the primary mechanism responsible for Adhsp 70i-enhanced oncolysis. Possible mechanisms for this finding may stem from the timely hsp 70 induction by Ad, considered important for intracellular Ad life cycle. Specifically, both Ad5 infection and heat shock induce stabilization of the filamentous actin network (17), and hsp 70 both promotes import of viral particles and colocalizes in the nucleus with Ad E1a (18). Furthermore, specific induction of hsp 70 by E1A during a lytic adenoviral infection is well characterized (3, 19). Viral DNA replication may also depend on hsp, as was documented for bacterial DnaK and DnaJ that are essential for bacteriophage DNA replication (20). A role of their human homologues hsp 70 and hsp 40 for small DNA tumor viruses has been described recently.

Specifically, because the J domain of hsp 40 usually stimulates the ATPase activity of hsp 70, it binds to and multimerizes the HPV E1 protein in the process of replication initiation (21) and is necessary for efficient SV40 DNA replication (22). hsp also play a role in binding denatured nuclear proteins and preventing their aggregation and disruption of nuclear matrix-dependent DNA replication and transcription. hsp 70 stimulates viral transcription in cells infected with measles virus and canine distemper virus (23). Whereas there is no direct evidence for hsp involvement in transcription of Ad genes, chaperones were reported to be involved in transcriptional regulation (24). Of note, despite the general host cell protein shut-off during the late phase of Ad infection, selective translation of hsp mRNA is maintained, thereby suggesting that synchronized expression of hsp in Ad-infected cells may confer a selective advantage for the viral life cycle (25). Ribosome shunting, the bypass of large mRNA segments before initiating translation at a downstream AUG, is directed by the Ad tripartite leader, and provides preferential translation to late Ad mRNA (26). During heat shock, the shunting mechanism is used exclusively by the hsp 70 mRNA. Elements within hsp 70 mRNA are related to those found in the Ad tripartite leader mRNA and share a unique property of promoting translation during the late phase of the Ad infectious cycle. Interestingly, elevated temperature can alleviate the defect of the Ad E1b M_r 55,000 mutant in mRNA transport, and the late gene expression and progeny production (27).

hsp 70 may also be involved at the late stages of Ad assembly, because its maximal synthesis concurs with the log phase of Ad structural assembly (28). This may be essential for virion production, because only 10% of structural Ad proteins synthesized are eventually assembled into virions (28).

hsp 70 protein has been found to be associated with Ad capsid, to colocalize with the Ad particles in the nucleus (3), and to associate with hexon, the major Ad capsid protein (29), and with the fiber

protein (28). The latter is the only Ad structural protein that requires glycosylation, where hsp 70 may direct its transport from the endoplasmic reticulum to the nucleus for virion assembly (30). The monomers of Ad5 hexon and fiber are insoluble and may need the cellular chaperone machinery to mediate, and perhaps regulate, their assembly into oligomeric complexes.

Another emerging pathway that may be related to Ad redirection of cellular machinery in the context of hsp is cellular transformation. For other small DNA tumor viruses, hsps have been found to play a major role in displacement of E2F from the Rb protein family. The SV40 large Tag requires an NH_2-terminal domain for cell transformation (21). This domain exhibits functional and sequence homology with the J domain of the bacterial DnaJ protein and the hsp 40 family. The NH_2-terminal domain of Tag interacts with hsp 70 and stimulates hsp 70-mediated disassembly of the Rb-E2F complex. Unlike SV40 and polyomavirus that execute an endogenous J domain in their Tag, HPV uses the host hsp 40. Because the LXCXE motif, mediating the binding to the pocket domain of the Rb protein family, is embodied in the Ad E1a and HPV E7 genes, it would be tempting to speculate that Ad may use the J domain of cellular hsp 40 to drive hsp 70-mediated Rb-E2F complex disassembly. Indeed, high levels of hsp 70 have been linked to early Ad gene expression in the absence of E1A (3).

In conclusion, this study demonstrates the induction of Ad5 burst and potentiation of its oncolytic effect by heat shock. Adhsp 70i infection also enhanced Ad-induced oncolysis but via a distinct, yet undefined mechanism.

Future implications of these findings may involve the use of hyperthermia to enhance the local oncolytic effect of Ad vectors *in vivo*, as well as additional exploration of a potential role of hsp in Ad-mediated oncolysis.

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A Model System for the Design of Armed Replicating Adenoviruses Using *p*53 as a Candidate Transgene

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Abstract

The therapeutic index of currently available modalities for most metastatic and locally advanced malignancies is low. Therefore, novel strategies have emerged to address this problem. Of these, cancer gene therapy endeavors to deliver genetic material efficiently and safely into tumor cells. However, despite promising pre-clinical results, replication-deficient viral vectors have demonstrated a limited efficacy in the clinical setting. To increase vector efficiency, replication-competent viruses have been proposed. Clinical trials have shown the safety of locally-injected, conditionally-replicative adenoviruses (CRAd), but have underscored the need for improved potency. To increase furthermore the therapeutic effect of CRAds, armed therapeutic adenoviruses (ATV) have been recently used *in vitro* and *in vivo*. However, constitutive transgene expression by replicating viral vectors may interfere with cellular signaling and viral production. Consequently, studies are equivocal in regard to the potential benefit of transgene expression by replication-competent adenoviral (Ad) vectors. To this end, we have designed an *in vitro* model system whereby transcomlpementation of replication-deficient Ad encoding *p*53 by the adenoviral E1 gene resulted in dramatic augmentation of cell killing and circumvented resistance to apoptosis induction. A complete correlation between the burst kinetics of replicating Ad and cell killing was not demonstrated in the context of our *p*53-ATV model. We further demonstrated that both Ad5 E1B-55kDa and E4-orf6 genes are required to enhance the cell killing effect by replicating Ad. We conclude that our ATV model system may be used for rational selection of candidate transgenes in the context of ATV.

Introduction

Gene therapy has been suggested as a novel strategy to improve the therapeutic index of cancer therapy. Whereas replication-deficient viral vectors have demonstrated great promise as anti-cancer agents in preclinical studies, this has not been translated into patient benefit in the clinical context(1). As a natural extension, replication-competent viruses have been suggested as a means to address the mutidimensional biological aspects of tumors(2). To date, replication-competent viruses employed in cancer clinical trials have included adenoviruses (Ad) and to a lesser extent, herpes simplex viruses (HSV). While replication-competent vectors have been shown to be safe, and potentially beneficial for therapy of localized tumors, their potency clearly needs to be improved(3). Therefore, 'armed' replicative viruses, incorporating therapeutic transgenes, have been introduced for cancer gene therapy(1, 4, 5). These armed therapeutic viruses (ATV) embody two potential advantages. First, they exhibit a capacity for up to 2000-fold higher levels of transgene expression relative to their replication-defective vector counterparts, in selected instances(1). Second, incorporated transgenes may provide a fail-safe mechanism to abolish viral replication by the induction of toxic cell death(6). There are, however, potential limitations to the use of ATV. In this regard, constitutive gene expression by Ad vectors may interfere with cellular signaling and result in cellular toxicity. Consequently, early apoptosis may impair viral replication(7), confounding anti-tumor effects linked to oncolysis.

Accordingly, results are inconclusive in regard to the utility of ATV. In three studies the inclusion of suicide/prodrug gene therapy with HSV thymidine kinase/gancyclovir (HSVtk/GCV) in a replicating Ad did not augment antitumor efficacy *in vitro* or *in vivo*(8-10). In contrast, other studies have shown that combined oncolysis, caused by a replicating virus and suicide/prodrug gene therapy with HSVtk/GCV, are complementary in improving outcome *in vivo*(4, 5). Furthermore, a replicating Ad with double suicide gene therapy containing the cytosine deaminase/5-FC (CD/5FC) and HSVtk fusion gene, markedly enhanced the cytopathic effect relative to the isolated viral effect(6, 11).

To address these inconsistencies, we have developed a strategy that induces replication of transgene-expressing, replication-deficient Ad vectors. As a proof of principle, we have selected a replication-deficient Ad vector encoding p53. Because the inhibition of viral replication by HSVtk/GCV or CD/5FC may counterbalance the therapeutic effect of ATV(12), p53 may be a useful therapeutic transgene in the context of ATV as it does not interfere with Ad replication(13), and may even increase its cytolytic effect(14). Furthermore, selection of *p*53 to indirectly induce apoptosis may circumvent transgene effects that induce apoptosis downstream of *p*53, and thus do not allow effective production, and lateralization, of the adenoviral vector(15, 16).

To this end, we induced replication of Ad vector encoding *p*53 by a variety of Ad mutants and evaluated cell killing and viral kinetics. Our studies show that ATV has a potential for higher cancer cell killing rates *in vitro* in the context of a transgene which is not counterproductive for viral replication. We further observed that the burst of replicating Ad does not necessarily correlate with cell killing in the context of ATV.

Finally, we found that the Ad E1B-55kDa and E4 orf6 genes are essential for the enhanced therapeutic effect of ATV encoding *p*53. These findings are highly consequential for an understanding of the efficacy of replicating Ad agents and for the design of ATV.

Materials and Methods

Recombinant Adenoviruses. A replication-deficient adenoviral expression vector for the delivery of the wild type human *p*53 cDNA has been reported previously (17). This vector expresses the human wild-type *p*53 under the transcriptional control of the CA promoter comprising a CMV enhancer and chicken β-actin promoter (AdCA*p*53). Ad338 is an Ad5 mutant lacking 524 base pairs within E1B-55 kDa gene(18). Ad355 is deleted for the E4 orf 6 gene (19). As a control for AdCA*p*53, we employed Ad5luc1, a replication-deficient Ad5, E1/E3 deleted, expressing the luciferase gene from the E1 region. As a wild-type equivalent we used Ad5luc3, a replication-competent Ad5, E1 intact, E3 deleted, expressing the luciferase gene from the E3 region. Both these viruses were constructed and propagated in our lab.

Cells, transfections and infections. A549 and H460, human lung cancer cell lines with intact p53, were obtained from the American Type Culture Collection (Manassas, VA). All cell lines were grown at 37 C° in RPMI 1640 medium with 2 mM L-glutamine, supplemented by 10% fetal bovine serum. Infections were performed 24 h after seeding $2x10^5$ cells per well in 12-well plates. For infections, growth medium was replaced by serum-free medium with the index virus at the indicated multiplicity of infection (MOI). An hour later, the infection media was removed, cells were rinsed with PBS, and 5% fetal bovine serum growth media was restored. The media was not replaced thereafter during the experiment and was sampled daily for determination of Ad5 E1 or E4 gene copy numbers.

For transient transfections, cells were seeded on 12-well plates and separately transfected with 1 μg of the indicated plasmids at a confluency of about 70%, using the Superfect (Qiagen, Santa Clarita, CA) method according to the instructions of the manufacturer. Expression vectors used for transfection were constructed as follows; pCMVE1 was derived from the shuttle plasmid (pShuttle) of the "Adeasy" system(20), by cloning the consecutive Ad E1 region extending from position 489 to 5789 of the Ad genome into the multicloning site, thereby deleting the right arm of pShuttle. Next, the CMV promoter/enhancer was cloned into the XhoI and EcoRV restriction sites of the recombinant plasmid. pCMVLuc was derived from cloning of the CMV promoter/enhancer into the mammalian expression vector pGL3 basic vector (Promega, Madison, WI) upstream of the luciferase gene.

TaqMan PCR assay. E1a copy number was determined for each medium sample obtained as of the first day after infection. Genomic DNA was isolated and cleaned using a Qiagen Tissue Kit (Qiagen, Santa Clarita, CA) following instructions of the manufacturer. Concentration of isolated DNA was determined by spectophotometry. TaqMan primers and probe design; the forward primer, reverse primer, and 6-FAM labeled probe to amplify the E1a and E4 genes, were designed by the Primer Express 1.0 software (Perkin Elmer, Foster City, CA) following the recommendations of the manufacturer. The sequences of the forward and the reverse E1a primers were AACCAGTTGCCGTGAGAGTTG (anneals between 966 and 986) and CTCGTTAAGCAAGTCCTCGATACAT (anneals between residues 1033 and 1009), respectively, while the TaqMan probe was CACAGCCTGGCGACGCCA (anneals between residues 988 and 1006).

The sequences of the forward and the reverse E4 primers were GGAGTGCGCCGAGACAAC (anneals between residues 816 and 833 of the E4 orf6 open reading frame) and ACTACGTCCGGCGTTCCAT (anneals between residues 883 and 865), respectively. The sequence of the TaqMan probe was TGGCATGACACTACGACCAACACGATCT (anneals between residues 836 and 863). With optimized concentration of primers and probe, the components of Real-Time PCR mixture were designed to result in a master mix with a final volume of 10 µl per reaction containing 1X Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 100 nM forward primer, 100 nM reverse primer, 1nM probe and 0.025% BSA. For the assay, 1µl of extracted DNA sample was added to 10 µl of PCR mixture in each reaction capillary. A no-template control received 10 µl of reaction mixture with 1 µl of water. All capillaries were then sealed and centrifuged using LC Carousel Centrifuge (Roche Molecular Biochemicals, Indianapolis, Indiana) to facilitate mixing. All PCR reactions were carried out using a LightCycler™System (Roche Molecular Biochemicals, Indianapolis, Indiana). The thermal cycling conditions were 10 minutes at 95 °C and 40 cycles of 15 seconds at 95 °C and 1 minute at 60 °C.

Statistical analysis

Data was initially tested for normality by the Shapiro-Wilk test. All abnormal tests were further tested for significance by the Wilcoxon-scores test. Results are expressed as a mean of at least three samples. Results were considered statistically significant for $p<0.05$.

Results

Induction of replication of AdCA*p*53 by E1 transfection to enhance cell killing by armed replicating Ad vector

Expression of *p*53 has been reported to be detrimental for Ad replication and cellular transformation(21), suggesting that Ad E1b55kDa mutants may not grow in normal tissues(22). In contrast, it has also been shown that *p*53 is essential for productive Ad infection(14) and that *p*53 overexpression does not interfere with Ad replication(13). Based on these considerations, we first determined the effect on viral replication and cell killing of heterologous *p*53 expression. In a previous study we have confirmed that a replication-deficient Ad vector expressing *p*53, AdCA*p*53, induces apoptosis and inhibits the growth of lung cancer cell lines *in vitro* and *in vivo* (17).

To evaluate the effect of E1 transfection on AdCA*p*53 replication and cell killing, we first confirmed that at an MOI of 20 pfu/cell, AdCA*p*53 does not replicate or cause significant cell killing. Specifically, A549 cells infected with either the replication-defective Ad5luc1 or with AdCA*p*53, remained viable for more than twelve days post infection. After this period, cells began to degrade but did not manifest any overt signs of viral cytopathic effect (CPE). Additionally, Ad E1a gene copy levels, as determined by quantitative PCR, were at the background level (data not shown). These results indicate that AdCA*p*53 does not replicate and does not cause a significant CPE in A549 cells at an MOI of 20. Next, we induced replication of AdCA*p*53 by transcomplementation with an intact adenoviral E1 gene. For this study, A549 cells were plated in each well of 12-well plates. After reaching 70% confluency, cells were transfected in triplicates with either pCMVE1 or pCMVluc.

Twenty-four hours later, cells were infected with either Ad5luc1 or AdCA*p*53 at an MOI of 20. Advanced CPE was observed three days after infection only for the E1-transfected, AdCA*p*53-infected cohort (figure 1A). Transfection with pCMVLuc, followed by infection with AdCA*p*53 did not induce any CPE, while transfection with pCMVE1 followed by infection with Ad5luc1 resulted in delayed and low CPE relative to E1-transcomplemented AdCA*p*53. These data clearly show that in our *p*53-ATV model, cell killing by *p*53 overexpression following induction of replication of AdCA*p*53, is more efficient relative to Ad-mediated oncolysis, or relative to cell killing of non-replicating AdCA*p*53. Because previous studies have shown that transgene expression may impair viral oncolysis, we next evaluated the kinetics of viral replication and burst relative to CPE. To this end, we assayed Ad E4 gene copies in the medium and found that kinetics of Ad DNA accumulation in the medium of E1-transfected, AdCA*p*53-infected cells indicated that viral replication was similar to the viral replication of E1-transfected, Ad5luc1-infected cells (figure 1B). Therefore, *p*53 overexpression by AdCA*p*53 does not inhibit or support Ad replication and burst. These patterns were corroborated by similar intracellular Ad DNA levels (data not shown). Thus, the enhanced killing effect of E1-transcomplemented AdCA*p*53 is not caused by Ad replication and burst from the cells but rather from transgene overexpression.

Intact E1 and E4 transcomplementation via replicative Ad are required to enhance the cell killing effect of AdCA*p*53

To further evaluate the enhancement of cell killing by AdCA*p*53 in the context of replicating Ad, we transcomplemented AdCA*p*53, or Ad5luc1, with a wild-type equivalent Ad vector Ad5luc3, or with Ad vectors deficient in either the E1B 55kDa or the E4 orf6 genes.

We reasoned that evaluation of distinct deletions of the Ad genome would allow the identification of Ad genes that are essential to achieve enhanced cell killing in the context of ATV. First, we validated that transcomplementation of intact E1 AdCA*p*53 with E1 expressed by a replicating Ad also augments cell killing of AdCA*p*53. A549 cells were infected with AdCA*p*53, or Ad5luc1, at MOI of 20. Forty-eight hours later cells were infected with Ad5luc3 at an MOI 5. Three days following infection with Ad5luc3, CPE was evident only for cells co-infected with AdCA*p*53 and Ad5luc3, while CPE for cells co-infected with Ad5luc1 and Ad5luc3 was delayed (figure 2A). To confirm the cell killing potency of AdCA*p*53 transcomplemented by a replicating virus, we performed the same experiment with the human lung adenocarcinoma H460 cell line. These cells have a significantly lower infectivity rate by Ad and may also be relatively resistant to apoptosis induced by the replication-deficient AdCA*p*53(17). We infected H460 exactly as above in figure 2A, and found that AdCA*p*53 transcomplemented by Ad5luc3, also induced a highly efficient cell killing. As in A549 cells, transcomplementation of Ad5luc1 by Ad5luc3 resulted in delayed CPE while AdCA*p*53 had no effect at this MOI (data not shown). Thus, transcomplementation with E1 of an adenoviral vector encoding *p*53 circumvents cellular resistance to induction of apoptosis by *p*53.

To further evaluate the possible roles of Ad genes in the potentiation of the therapeutic effect of AdCA*p*53, we evaluated the interaction of an Ad vector deleted of the E1B 55kDa gene. To this end, we infected A549 with AdCA*p*53, or Ad5luc1, as above in figure 2A. After 48 hours cells were co-infected with the E1B 55kDa-deleted Ad338 at an MOI of 5. Plates were stained with crystal violet after the observation of advanced CPE (figure 2B).

In this instance transcomplemenation of AdCA*p*53 by Ad338 was distinct from transcomplementation by the E1-intact Ad5luc3 in two ways. First, CPE was observed with Ad338 transcomplementation of AdCA*p*53 only as of the fifth day, two days after observation of CPE following Ad5luc3 transcomplementation of AdCA*p*53. Second, there was no difference in the cell killing patterns of AdCA*p*53 or Ad5luc1 following co-infection with Ad338. Thus, deletion of the E1B 55kDa gene prevents the enhancement of cell killing observed for transcomplementation of AdCA*p*53 by an intact E1 gene. Because the Ad E1B 55kDa protein functions in concert with the E4 orf6 gene product during the late Ad infection phase, we hypothesized that the latter is also essential to augment the effect of AdCA*p*53 in the context of ATV. To this end, we infected A549 cells with AdCA*p*53, or Ad5luc1, as above in figure 2A. Forty eight hours later, we co-infected with the E4 orf6-deleted Ad355 at an MOI of 5. As for Ad338, transcomplementation of AdCA*p*53 with E4 orf6-deleted Ad355 did not induce cell killing further than that achieved by Ad oncolysis (figure 2C). Thus, these studies show that intact E1 and E4 are required to enhance the cell killing effect of AdCA*p*53.

Enhancement of AdCA*p*53-induced cell killing is related to replication and burst kinetics of the transcomplementing Ad vectors

One interpretation of the above studies would be that in this model system of *p*53-ATV, AdCA*p*53 transcomplementation by E1 results in dramatically higher cell killing by virtue of transgene expression. Alternatively, it could be asserted that *p*53 overexpression supports the oncolytic effect of Ad5luc3, thereby inducing Ad burst as a primary cause of cell death. To address this issue, we assayed the burst kinetics of Ad5luc3, Ad338 and Ad355.

Because both the replication-competent and replication-incompetent Ad vectors we had studied contain functional E4 sequences, we selected E1a gene as an indicator of the replication and burst kinetics of the various replication-competent transcompelmenting Ad vectors. To this end, we sampled daily the media for E1a gene copies from the wells infected for the cell killing assays. While media sampling is a direct method to evaluate viral burst of replication-competent viruses(2), it indicates replication only indirectly. The various kinetics of viral burst are described in figure 3A. When evaluating the kinetics of the transcomplementing vectors, Ad5luc3, Ad338 and Ad355, without the effect of heterologous *p*53 overexpression, the levels of Ad5luc3 in the media were the highest as of the first day after infection. Consequently, the wild-type equivalent Ad5luc3 replicates and bursts efficiently. In contrast, Ad355 levels were lower than Ad5luc3, and Ad338 levels were the lowest. These patterns are in accord with the potency of these viruses in transcomplementing AdCA*p*53-mediated enhanced cell killing. Specifically, only the wild-type equivalent Ad5luc3 could achieve both high replication and burst rates, and enhance the cell killing of AdCA*p*53. Ad 355 and ad338 had lower rates of replication and burst, correlating with their incompetence in enhancement of AdCA*p*53-mediated cell killing. However, a striking finding of the evaluation of Ad5luc3 burst, with or without AdCA*p*53, was that despite the clear augmentation of cell killing when co-infecting with AdCA*p*53, the kinetics of Ad5luc3 burst did not differ significantly from the burst kinetics assayed for co-infection of Ad5luc3 with Ad5luc1. A trend towards earlier burst of Ad5luc3 was observed following co-infection of Ad5luc3 with AdCA*p*53, which was distinctly different from the unequivocal cell killing enhancement induced by co-infection with these two viruses.

This discrepancy is even more striking when evaluating the kinetics of Ad388 and Ad355 during co-infection with either AdCA*p*53 or Ad5luc1. Both these viruses had remarkably similar burst kinetics when co-infecting with either AdCA*p*53 or Ad5luc 1 (figure 3A), and had comparable viral DNA replication (figure 3B). The similarities of viral replication and burst for these Ad mutants are in accord with their cell killing properties, as co-infection with AdCA*p*53 did not influence either cell killing (figure 2B,2C) or viral kinetics (figure 3A, 3B). These findings further underscore the discrepancy of the enhanced cell killing effect of AdCA*p*53 transcomplemented by intact E1 expression by Ad5luc3 or following transfection. Because the differences in burst kinetics cannot explain the enhanced cell killing, timely and efficient transgene expression by the transcomplemented Ad vector AdCA*p*53 may account for the augmented cell killing following E1 expression with transfection, or by replication-competent Ad. Thus, intact E1 and E4 are required to enhance the cell killing effects of AdCA*p*53.

Discussion

A major limitation of cancer gene therapy is the inadequacy of replication-deficient Ad vectors to efficiently infect target tumor cells(23). To address this problem, replicating vectors have been suggested as a means to amplify an initial infection event(24). However, despite the safety of these viruses deriving from tumor selectivity of replication dynamics, their efficacy is limited(3). Whereas incorporation of a therapeutic transgene into a replicating Ad, to form an ATV, has the potential to enhance the potency of replication-competent vectors, recent studies could not resolve this issue. Specifically, expression of incorporated transgenes may directly compromise the goal of viral replication, and thus, indirectly interfere with anti-tumor oncolysis(25).

In this study, we hypothesized that alleviation of the replication-restriction on AdCA*p*53 would form a platform for studying the therapeutic effects and viral kinetics of ATV. First, we validated that intact E1 induces replication of AdCA*p*53, and that Ad replication is not affected by *p*53 overexpression. These findings validated the recent documentation of Ad replication despite *p*53 overexpression(13). Next, we showed that induction of replication of AdCA*p*53, mimicking an ATV, results in a higher therapeutic effect than replicating Ad with no transgene. However, this effect depended on Ad transcomplementation by intact E1 and E4. This finding questions the utility of ATV derived from E1B 55kDa-deleted or E4 orf6-deleted Ad genomes, at least for vectors encoding *p*53. Because previous studies have shown that ATV with HSVtk/GCV are not more oncolytic than replicating Ad, stringent selection of the therapeutic transgene is clearly essential for the design of ATV.

In this regard, our ATV model allows screening of therapeutic transgenes *in vitro*. Careful scrutiny of transgenes according to this model may select for ATV expressing transgenes that are not counterproductive. Another interesting finding of our studies was the dissociation of Ad5luc3 burst from the cell killing in the context of *p*53 overexpression. While the transgene from ATV induced slightly earlier burst of the co-infecting replicative Ad, cell killing depended primarily on the functionality of E1 transcomplementation of AdCA*p*53. In this regard, we found that the Ad E1B-55kDa and E4 orf6 genes are both essential for the enhanced therapeutic effect of ATV, at least for AdCA*p*53.

In conclusion, we have developed an *in vitro* model for the evaluation of therapeutic transgenes in the context of ATV. A replication-deficient Ad vector encoding *p*53 was induced to function as an ATV and was shown to be superior to replicative Ad by virtue of its cell killing effect. In view of the need to improve vector efficacy for cancer gene therapy, we suggest this model system to allow for a rational design of ATV.

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Figure Legends

Figure 1. Transfection of the adenoviral E1 gene enhances the cell killing effect of AdCA*p*53 in an oncolytic-independent fashion. A. The human lung adenocarcinoma cell line A549 was transfected in triplicates with the adenoviral E1 expression vector pCMVE1 or with a control plasmid expressing luciferase pCMVLuc. Twenty-four h later, cells were infected with E1-deleted, replication-incompetent adenoviral vectors at an MOI of 20 pfu/cell. Vectors studied included a control vector expressing the luciferase reporter gene (Ad5luc1) and a p53 expressing vector (AdCA*p*53). Cells were stained with crystal violet after observation of advanced cytopathic effect (CPE). **B.** Analysis of replication of adenoviral vectors induced by E1 transcomplementation. A549 cells were transfected with pCMVE1 or pCMVLuc and infected 24 h later with Ad5luc1 or AdCA*p*53, exactly as in figure 1A. Media samples were collected in triplicates from the different cohorts and subject to quantitative polymerase chain reaction (PCR) analysis of adenoviral E4 copy number as an index of adenoviral replication and burst. Experimental groups included pCMVE1+Ad5luc1 (▲), pCMVE1+AdCA*p*53 (■), and pCMVLuc+AdCAp53 (◆).

Figure 2. Intact E1 and E4 genes are required for enhancement of cell killing effect by transcomplementation of AdCA*p*53 from a replicative Ad. A. Analysis of cell killing of E1-transcomplemented AdCA*p*53 via the wild-type equivalent replicating adenovirus, Ad5luc3. A549 cells were infected at an MOI of 20 with the p53 expressing, E1-deleted adenoviral vector AdCA*p*53 or the E1-deleted control virus Ad5luc1.

Forty-eight h later, cells were infected with the E1-expressing adenovirus Ad5luc3 at an MOI of 5. Cells were stained with crystal violet after observation of advanced CPE. M represents mock-infected cells. **B.** Analysis of cell killing of AdCA*p*53 after transcomplementation with the E1B 55kDa-deleted adenovirus Ad338. A549 cells were infected at an MOI of 20 with the p53 expressing, E1-deleted adenoviral vector AdCA*p*53 or the E1-deleted control virus Ad5luc1. Forty-eight h later, cells were infected with the E1B 55kDa-deleted adenovirus Ad338 at an MOI of 5. Cells were stained with crystal violet after observation of advanced CPE, that was delayed relative to the CPE induced by AdCA*p*53 transcomplemented by Ad5luc3 expressing the intact E1 gene (figure 2A). M represents mock-infected cells. **C.** Analysis of cell killing of AdCA*p*53 after transcomplementation with the E4 orf6-deleted adenovirus Ad355. A549 cells were infected at an MOI of 20 with the *p*53 expressing, E1-deleted adenoviral vector AdCA*p*53 or the E1-deleted control virus Ad5luc1. Forty-eight h later, cells were infected with the E4 orf6-deleted adenovirus Ad355 at an MOI of 5. Cells were stained with crystal violet after observation of advanced CPE. M represents mock-infected cells.

Figure 3. Wild-type equivalent Ad and E1B and E4 mutant Ad vectors differ in their replication and burst kinetics. Analysis of replication of adenoviral vectors as a function of E1 and E4 status, and as a function of *p*53 overexpression. A549 cells were infected with AdCA*p*53 or Ad5luc1 at an MOI of 20. Forty-eight h later, cells were infected with Ad5luc3, Ad338 or Ad355. Media samples were collected daily in triplicates from the different cohorts and subject to quantitative PCR analysis of Ad E1a copy number as an index of Ad replication and burst.

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in the final step, instead of FDA, cells were stained with 5 μM YO-PRO-1 iodide (quinolinium, 4-{(3-methyl-2(3H)-benzoxazolylidene)methyl}-1{(3(trimethyl-ammonio) propyl}-, diiodide; Molecular Probes, Eugene, OR). YO-PRO-1 is a nucleic acid stain that emits green fluorescence, which passes through the plasma membranes of apoptotic cells even when they are still viable, *i.e.*, when they have undergone changes in membrane permeability but no major membrane breaks (29 , 30) . Excitation by the red diode (535 nm) and the laser argon beam (480 nm) was used for Cy5 and YO-PRO-1 iodide, respectively. Cells with reduced forward scatter or strong side scatter (dead cells) and negative W6–32 staining (murine cells) were gated out. The remaining YO-PRO-1-positive cells were assumed to be apoptotic, but still viable, NPC cells.

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Cancer Research 59, 924-930, February 15, 1999]

Control of Apoptosis in Epstein Barr Virus-positive Nasopharyngeal Carcinoma Cells: Opposite Effects of CD95 and CD40 Stimulation

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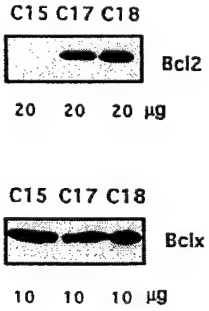
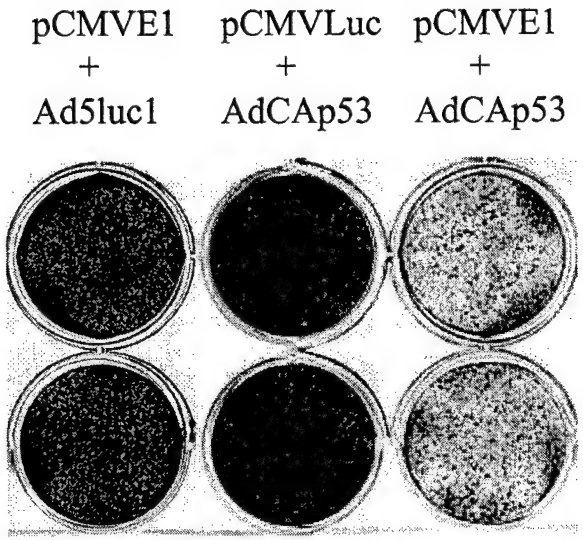


Fig. 8. Bcl-2 and Bcl-x synthesis in NPC tumor lines. The polyclonal antibody used to detect *Bcl-x* recognized both the large and the small forms of the protein. Only the large form (Bcl-x_L) was detected. On a longer exposure, a faint *Bcl-2* band was detected in *C15* material.

A.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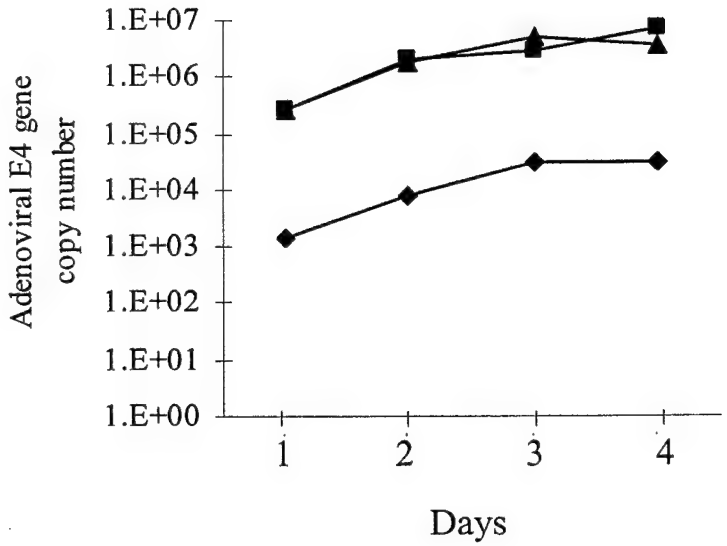
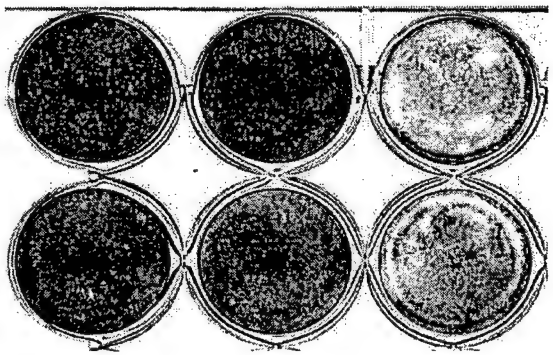
+
Ad5luc1
pCMVLuc
+
AdCAp53
pCMVE1
+
AdCAp53

B.


Adenoviral E4 gene copy number
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01
1.E+00
1
2
3
4
Days

A.

M | Ad5luc3 + Ad5luc1 | Ad5luc3 + AdCAp53



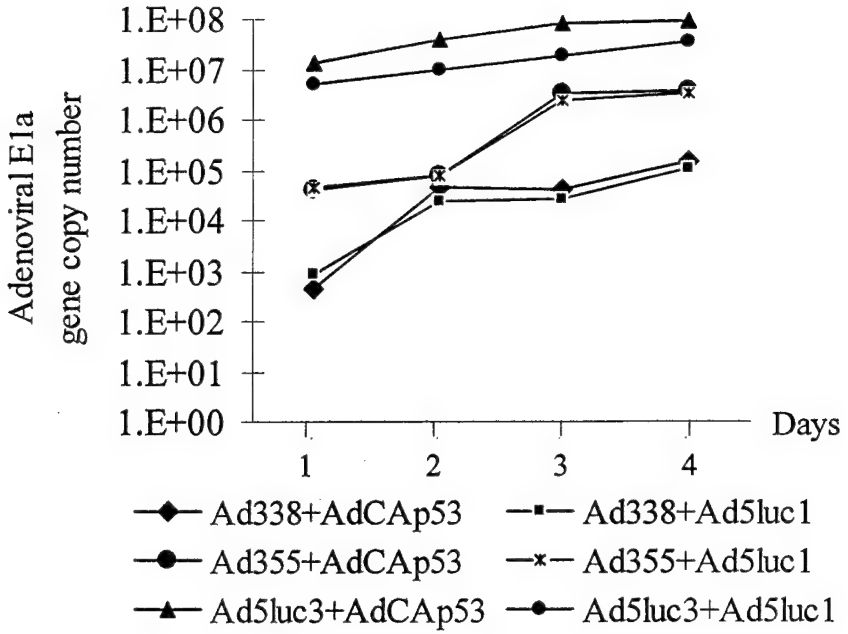
B.

M | Ad338 + Ad5luc1 | Ad338 + AdCAp53

C.

M | Ad355 + Ad5luc1 | Ad355 + AdCAp53

A.


Adenoviral E1a gene copy number
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1.E+06
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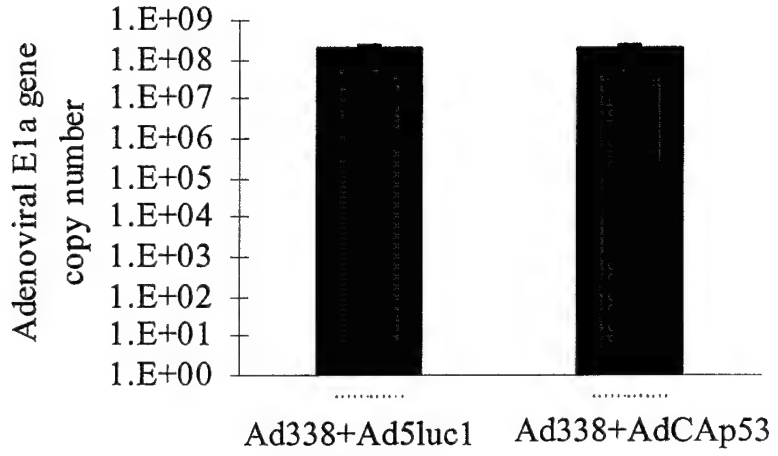
1.E+04
1.E+03
1.E+02
1.E+01
1.E+00
Days
1
2
3
4
Ad338+AdCAp53
Ad338+Ad5luc1
Ad355+AdCAp53
Ad355+Ad5luc1
Ad5luc3+AdCAp53
Ad5luc3+Ad5luc1

B.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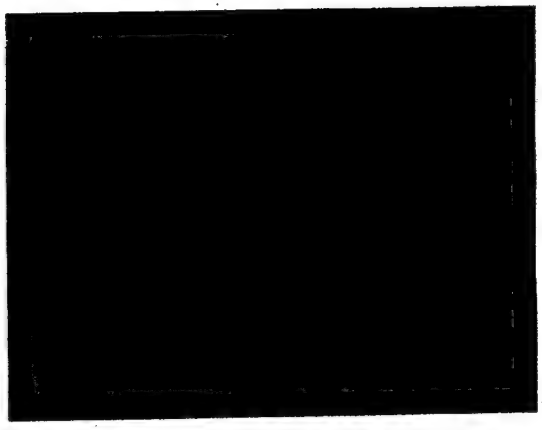
1.E+02
1.E+01
1.E+00
Ad338+Ad5luc1
Ad338+AdCAp53

AdCAp53
+
Ad338

Ad5luc3
+
Ad5luc1

AdcAp53
+
Ad5luc3



[CANCER RESEARCH 61, 6377–6381, September 1, 2001]

Advances in Brief

Targeting Oncolytic Adenoviral Agents to the Epidermal Growth Factor Pathway with a Secretory Fusion Molecule[1]

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Abstract

Cancer gene therapy with conditionally replicating adenoviruses is a powerful way of overcoming low tumor transduction. However, one of the main remaining obstacles is the highly variable level of the coxsackie-adenovirus receptor expression on human primary cancers. In contrast, the epidermal growth factor receptor (EGFR) is overexpressed in various tumor types, and its expression correlates with metastatic behavior and poor prognosis. We constructed an adenovirus expressing a secretory adaptor capable of retargeting adenovirus to EGFR, resulting in a more than 150-fold increase in gene transfer. A replication-competent dual-virus system secreting the adaptor displayed increased oncolytic potency *in vitro* and therapeutic gain *in vivo*. This approach could translate into increased efficacy and specificity in the treatment of EGFR overexpressing human cancers.

Introduction

CRADs[3] are a promising and novel way of overcoming low tumor transduction, which is the main obstacle preventing effective gene transfer and therapeutic effect in clinical applications of cancer gene therapy (1). However, one of the main reasons why the unparalleled transduction efficacy of Ads has not translated into similar results in humans is the variable level of the CAR on primary cancers (2–9) *in vivo*. CAR is ubiquitously expressed on normal epithelial tissues and is the main receptor mediating binding of the most commonly used Ad serotypes 2 and 5. Expression of CAR may be the major factor determining the rate of transduction (4, 6, 9–11). Importantly, recent evidence (11) suggests that CAR expression may inversely correlate with the malignant potential of tumors, resulting in low infectivity of highly aggressive tumors. In contrast to the expression profile of CAR, EGFR, the prototype of cancer-associated receptors, is commonly overexpressed in many if not most carcinomas with correlation to metastatic behavior and poor prognosis (12). A powerful approach for increasing tumor transduction could be combining the tissue-penetrating capability of CRADs with the transductional control provided by retargeting moieties. In support of this hypothesis, an artificial receptor system has been used to demonstrate that the effect of Ad dispersion and subsequent oncolysis critically depends on receptor expression (13). We have constructed a novel virus that mediates secretion of a fusion molecule consisting of the extracellular domain of CAR and EGF. We then explored the capability of the sCAR-EGF to retarget Ad to EGFR. Finally, we demonstrated that infection of cancer cells with a sCAR-EGF-retargeted replication-competent dual-virus system resulted in increased oncolysis *in vitro* and a therapeutic benefit *in vivo*.

Materials and Methods

Viruses. For construction of AdsCAR-EGF, a replication-deficient Ad with sCAR-EGF in E1, the gene coding for sCAR-EGF was cloned from pFBshCAR-EGF (14) into pShuttle-CMV (Quantum, Montreal, Quebec, Canada). Homologous recombination with pAdEasy-1 (Quantum) was performed in *Escherichia coli*, followed by confirmation of structure with *Eco*RV and *Pac*I digestions, PCR, and sequencing of the transgene (data not shown). The viral genome was transfected into 293 cells for plaque purification, followed by cesium chloride purification and standard titering with OD260 and plaque assay. Resulting titers were 3.8×10^{11} VPs/ml and 1.0×10^{10} plaque-forming units/ml, ratio = 38.4 VPs/plaque-forming unit. Large-scale preparations of AdCMVLuc (a nonreplicating Ad-expressing luciferase; courtesy of Dr. Robert Gerard, Texas Southwestern Medical Center, Dallas, TX), AdsCAR6H (a nonreplicating Ad-mediating secretion of sCAR6H),[4] and Δ24 (an Ad with a 24-bp deletion in *E1A*, allowing selective replication in cells mutant in the *Rb-p16* pathway; Ref. 15) were performed with standard methods on 293 cells (or A549 cells for Δ24).

CRAdsCAR-EGF denotes a replication-competent, sCAR-EGF-secreting dual-virus system consisting of equal VPs of AdsCAR-EGF and Δ24 mixed immediately before administration to cells. CRAdCMVLuc is the respective combination of Δ24 and AdCMVLuc. Δ24 has been characterized previously (15). Validating the dual-virus strategy, it has been demonstrated that transcomplementation of E1 proteins from a plasmid or replication-competent virus results in replication of E1-deleted viruses present in the same cell (9, 16–18).

Cell Lines. 293 cells were purchased from Microbix (Toronto, Ontario, Canada). A549 (lung cancer), HeLa (cervical cancer), U118 (glioma), A431 (squamous cell skin cancer), BT474, and MB-453 (breast cancer) were obtained from the American Type Culture Collection (Rockville, MD), and SKOV3.ip1 cells (ovarian cancer) are from Dr. Janet Price (M. D. Anderson Cancer Center, Houston, TX). Cell lines were propagated in the recommended conditions.

Protein Detection. HeLa cells were infected overnight with 50 VP/cell, and BT474 and MB453 cells were infected with 500 VP/cell of AdsCAR-EGF, AdsCAR6H, and AdCMVLuc. Supernatants were collected at 48 h, and cellular debris was removed by centrifugation. Dilutions in a volume of 300 μl were transferred onto a nitrocellulose membrane using the Bio-Dot apparatus (Bio-Rad). BSA (3%) was used for blocking, followed by detection with a 1:5000 polyclonal mouse anti-CAR antibody (14) and 1:2000 goat antimouse alkaline phosphatase (Sigma Chemical Co., St. Louis, MO) in 3% BSA. Western blot analysis on the supernatants was performed with standard methodology in a 12% two-phase gel, and proteins were detected as above. Baculovirus-expressed and -purified sCAR-EGF and sCAR6H (14) were used as controls.

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[3] The abbreviations used are: CRAD, conditionally replicative adenovirus; Ad, adenovirus; CAR, coxsackie-Ad receptor; EGF, epidermal growth factor; EGFR, EGF receptor; sCAR-EGF, secretory CAR-EGF fusion molecule; CMV, cytomegalovirus immediate early promoter; VP, viral particle; sCAR6H, secretory CAR with 6-histidine tag.

[4] I. Dmitriev, unpublished observations.

Retargeting Assays. SKOV3.ip1, U118, and A431 cells were infected in duplicate with AdCMVLuc preincubated for 30 min with aliquots of supernatants (see above). Twenty VP/cell were used for SKOV3.ip1, whereas 200 VP/cell were used for U118 and A431. Luciferase assay was performed 48 h later (Luciferase Assay System; Promega, Madison, WI).

Cell-killing Assays. SKOV3.ip1 cells were plated in triplicate and infected with Δ24 or AdCMVLuc for 1 h. Aliquots (25 μl) of AdsCAR-EGF or AdCMVLuc supernatant (from protein detection assays) were added each day. At 20 days, cells were fixed with formalin and stained with crystal violet.

Next, A431 and SKOV3.ip1 cells were infected with the CRAdCMVLuc or CRAdsCAR-EGF for 1.5 h. After incubating for 20 days with medium changes, the remaining cells were fixed and stained as above.

Preliminary toxicity analysis was performed by infecting A431 and SKOV3.ip1 cells with 50, 100, 200, 500, and 1000 VP/cell of AdsCAR-EGF and AdsCAR6H, followed by medium changes every 2 days for 20 days.

Animal Experiments. Initially, A431 cells were infected *ex vivo* with 50 VP/cell of CRAdsCAR-EGF for 1 h followed by a 5-h incubation. Cells were then collected and mixed with uninfected cells, and a total of 10^7 cells were injected into flanks of athymic CD-1/nu mice (Charles River Laboratories, Wilmington, MA; n = five/group). Tumor size was determined as the mean of the shortest and longest diameter (to avoid variability attributable to difficulty with estimation of height).

To compare CRAdsCAR-EGF to CRAdCMVLuc *in vivo*, s.c. tumors were established by injecting 5×10^6 A431 cell into both flanks of athymic mice (n = five/group). When tumors were ~5 mm ("day 0"), viruses were injected intratumorally in a 15-μl volume and tumors were measured as above. Each mouse was checked daily for the absence of pain or distress (19).

Statistics. Upon termination of the experiment, mean tumor size and SDs were calculated for each group of animals for each time point. The nonparametric change-point test (20) was used to show a systematic change in the pattern of observations as opposed to fluctuation attributable to chance. The mixed model (21) was used to longitudinally model the data on each tumor. The variance covariance structure was determined by using Akaike's Information Criteria (22). The Proc Mixed procedure in SAS v.6.12 (SAS Institute, Cary, NC) was used to examine the effects of group and time on tumor growth.

Results

Infection of Cells with AdsCAR-EGF Results in Secretion of sCAR-EGF. Initially, infection of high EGFR HeLa cells (23) with AdsCAR-EGF produced no evidence of secretion, whereas sCAR6H was secreted (Fig. 1). With low EGFR-expressing cells MB453 and BT474 (14, 24), secretion of sCAR-EGF was detected. The amount of protein was estimated at 110 ng/ml (75-cm^2 flask; 12 ml of medium). Western blot confirmed secretion (Fig. 1*B*). The altered migration rate of the protein in comparison with baculovirus-expressed sCAR-EGF perhaps resulted from altered charge caused by different glycosylation by insect cells. A preliminary investigation on sCAR-EGF toxicity was performed by infecting SKOV3.ip1 and A431 cells with various amounts of AdsCAR-EGF and AdsCAR6H without significant differences in cell viability (data not shown).

Secreted sCAR-EGF Mediates Retargeting of Ad to EGFR. Aliquots of supernatant from AdsCAR-EGF-infected BT474 cells were incubated with AdCMVLuc. The virus-supernatant mix was used for infection of SKOV3.ip1, U118, and A431 cells, which display moderate (SKOV3.ip1 and U118) to high (A431) EGFR expression and moderate (A431) to low (SKOV3.ip1 and U118) CAR expression (4, 14). A supernatant dose-dependent increase in luciferase expression was seen, with the highest readings 17.1-, 20.2-, and 158-fold higher than without retargeting for SKOV3.ip1, U118, and A431 cells, respectively (curves with triangles in Fig. 2).

With the highest amounts of supernatant from AdsCAR6H-infected cells, luciferase expression was reduced to 73%, 48%, and 65% (on SKOV3.ip1, U118, and A431, respectively) of the highest values for the series (curves with squares in Fig. 2). sCAR6H binds to Ad fiber but does not mediate binding to EGFR, thus modeling blockage of CAR-binding with sCAR-EGF.

Retargeting Replication-competent Ad to EGFR Results in Increased Cell Killing *in Vitro*. To validate Δ24 replication in SKOV3.ip1 cells, infections were performed at 0, 0.01, 0.1, or 1 VP/cell, and aliquots of supernatant (from BT474 cells infected with

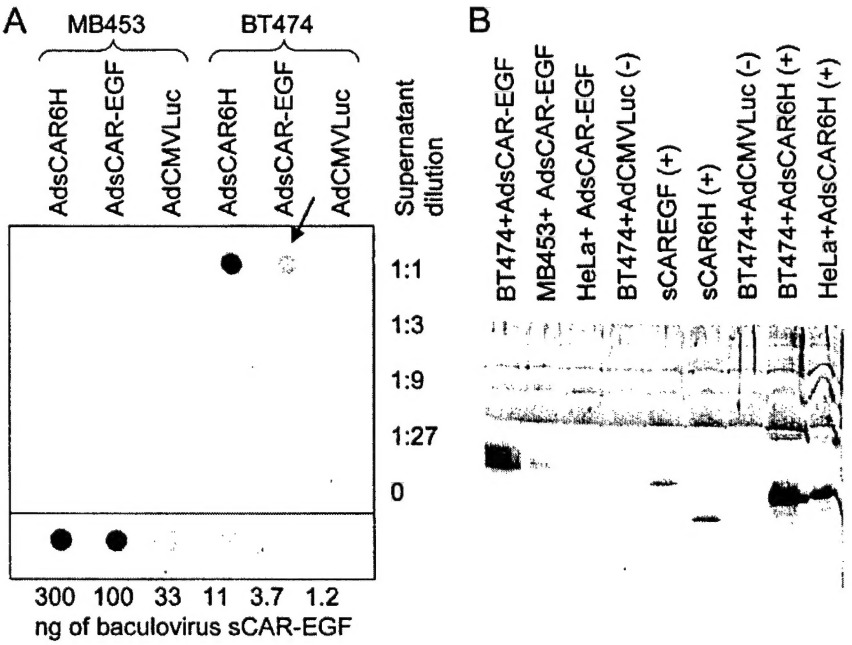

Fig. 1. Secretion of sCAR-EGF from human cancer cells infected with AdsCAR-EGF. *A*, supernatant from MB453 and BT474 (both low EGFR breast cancer lines) cells infected with AdsCAR6H (codes for human CAR ectodomain, positive control), AdsCAR-EGF, or AdCMVLuc (negative control) was centrifuged and then transferred onto a membrane. *Arrow*, the signal for sCAR-EGF. When compared with known amounts of sCAR-EGF (*lowest row*), the amount of secretion could be estimated at 110 ng/ml. *B*, Western blot suggested that the sCAR-EGF secreted from BT474 and MB453 cells (*Lanes 1–2*) was close in size to baculovirus-expressed sCAR-EGF (*Lane 5*). High EGFR HeLa cells (*Lane 3*) did not show evidence of sCAR-EGF secretion, but the positive control sCAR6H was secreted (*Lane 9*). −, supernatants collected from cells infected with AdCMVLuc (*Lanes 4 and 7*). These serve as negative controls. +, the positive controls, including *Lane 8*, which has supernatant from BT474 cells infected with AdsCAR6H. sCAR-EGF and sCAR6H (*Lanes 5–6*) are purified baculovirus-expressed proteins.

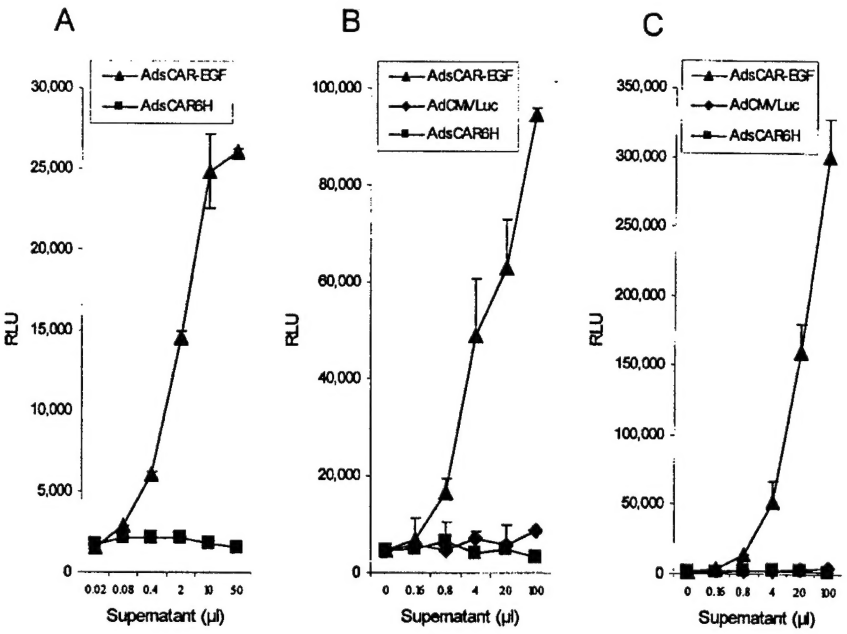

Fig. 2. Secreted sCAR-EGF mediates retargeting of Ad to EGFR. Increasing amounts of supernatant collected from cells infected with AdsCAR-EGF (curves with ▲) were incubated with AdCMVLuc, and then the mix was added to (*A*) SKOV3.ip1 (ovarian cancer), (*B*) U118 (glioma), or (*C*) A431 (squamous cell skin cancer) cells. These cells express moderate to high EGFR and thus resemble many aggressive human cancers. Supernatant from cells infected with AdCMVLuc (◆) or AdsCAR6H (■) were used as controls. Relative light units (*RLU*) are means of duplicate experiments (±1 SD). With the highest amounts of supernatant, luciferase readings were 17.1-fold (*A*), 20.2-fold (*B*), and 158-fold (*C*) higher with retargeting. The slope of the AdsCAR-EGF curves suggests that maximum retargeting potential was not reached.

AdsCAR-EGF or AdCMVLuc) were added daily. At 20 days, cell killing and partial loss of monolayer was seen only with cells that had been infected with 1 VP/cell and subjected to AdsCAR-EGF/BT474 supernatant (data not shown).

To study the effect of continuous sCAR-EGF secretion on the oncolytic potential of CRADs, we infected SKOV3.ip1 and A431 cells with the CRAdsCAR-EGF and CRAdCMVLuc dual-virus systems. On both cell lines, infection with CRAdsCAR-EGF resulted in cell killing with one to two orders of magnitude less virus than with CRAdCMVLuc (Fig. 3).

Targeting Replicative Ad to EGFR Results in a Therapeutic Advantage *in Vivo*. Various proportions of infected and uninfected A431 cells were mixed and injected s.c. (Fig. 4*A*). One percent of infected cells was sufficient to inhibit tumor growth, and 5% or more resulted in healing of tumors. None of the mice showed signs of illness or distress, suggesting that the secretion of sCAR-EGF did not cause overt toxicity.

To evaluate sCAR-EGF retargeting *in vivo*, CRAdsCAR-EGF or CRAdCMVLuc were administered with a single intratumoral injection into established A431 tumors (Fig. 4, *B–C*). The change-point test (20) revealed that the tumor growth pattern changed at 13 days for 10^9 VP CRAdsCAR-EGF ($P = 0.0045$), 21 days for 10^9 VP CRAdCMVLuc ($P = 0.0012$), 17 days for 10^8 VP CRAdsCAR-EGF ($P = 0.0026$), and 25 days for 10^8 VP CRAdCMVLuc ($P = 0.0011$), *i.e.*, 8 days earlier for CRAdsCAR-EGF with both doses.

The change-point test and the test of fixed effects (22) showed that there was a significant correlation between observations of tumor size and time ($P < 0.0001$ for all of the groups). A polynomial equation was fit for each group, thereby creating a mathematical model for each growth pattern ("modeled" in Fig. 4). The mixed model (22) was used

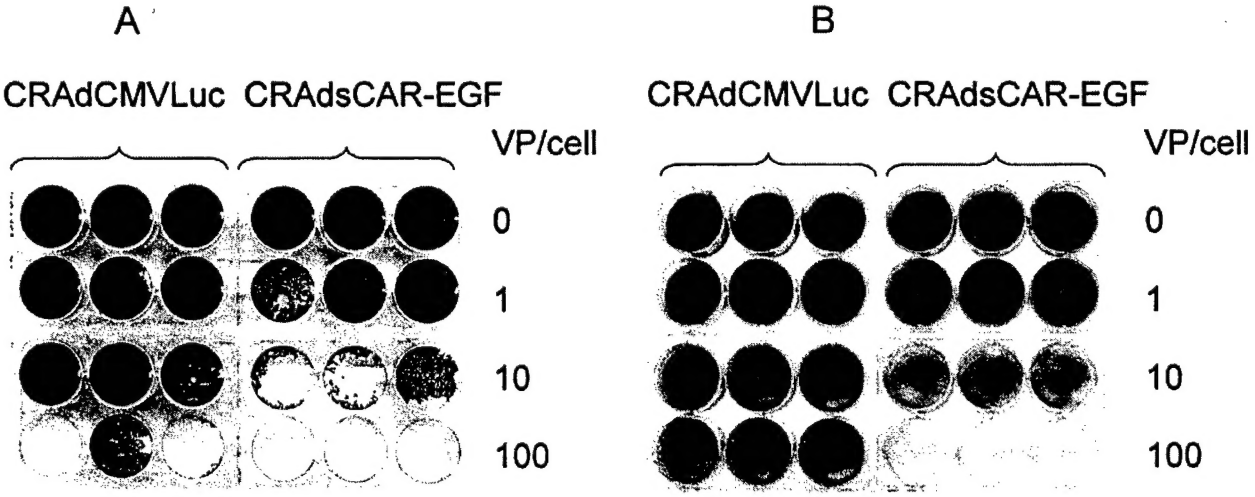

Fig. 3. Targeting oncolytic Ad to EGFR with sCAR-EGF results in an increased oncolytic effect. (*A*) SKOV3.ip1 or (*B*) A431 cells were infected with CRAdsCAR-EGF, a replication-competent sCAR-EGF-secreting dual-virus system. Oncolytic potency was compared with CRAdCMVLuc, which is isogenic in regard to replicativity but does not secrete a retargeting molecule. A similar effect on cells was observed with 1 VP/cell of CRAdsCAR-EGF as with 100 VP/cell of CRAdCMVLuc, suggesting increased oncolysis because of sCAR-EGF secretion-mediated EGFR targeting.

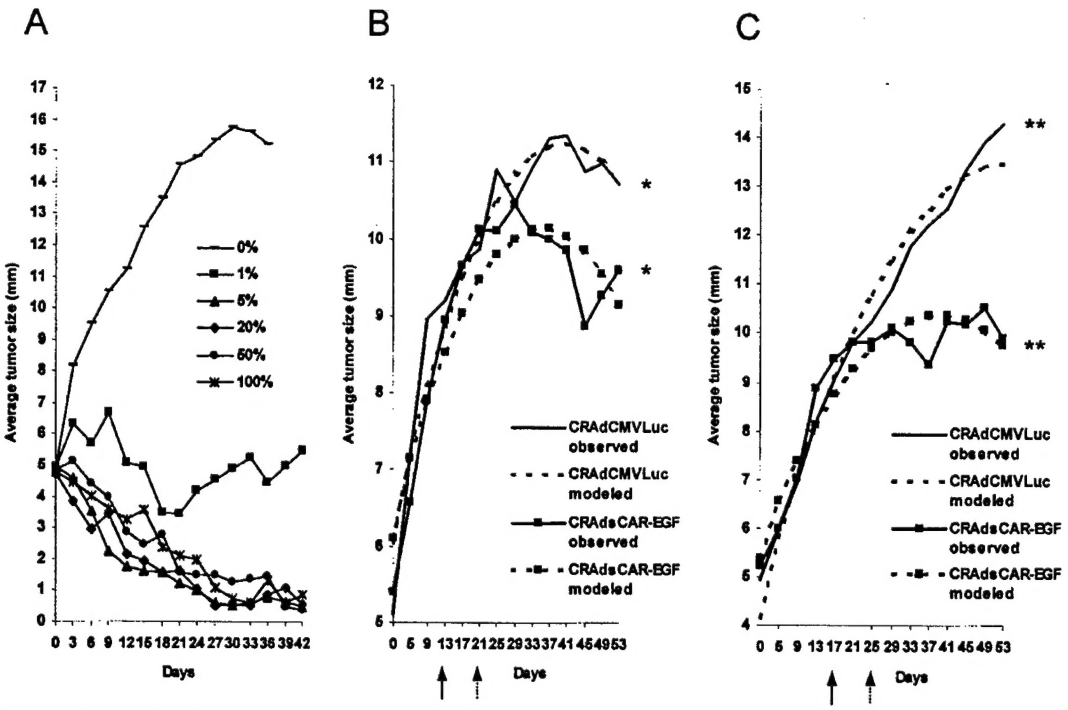

Fig. 4. sCAR-EGF secretion results in therapeutic efficacy *in vivo*. *A*, various percentages of A431 cells were infected *ex vivo* with CRAdsCAR-EGF and mixed with uninfected cells. One percent of infected cells was sufficient to inhibit tumor growth, whereas 5% or more resulted in healing of mice. Single intratumoral injections of (*B*) 10^9 or (*C*) 10^8 VP of CRAdsCAR-EGF or CRAdCMVLuc dual-virus systems were performed into established A431 xenografts. *Arrows* (*solid* for CRAdsCAR-EGF, *dotted* for CRAdCMVLuc) indicate the change-point in tumor growth characteristics, which represents the time point when oncolysis changes the initial pattern of tumor growth. On the basis of tumor size measurements (*observed*), the growth patterns were mathematically modeled for statistical comparison (*modeled*), and CRAdsCAR-EGF was found to be more oncolytic. *, $P < 0.05$; **, $P < 0.0001$.

to allow for the curvature of the plots, which was caused by the opposite effects of oncolysis and cell division. For the mice that received injections with 10^9 VP, groups were significantly different from day 29 onwards ($P < 0.05$; Fig. 4*B*). When 10^8 VP was used (Fig. 4*C*), the differences were significant from day 25 onwards ($P = 0.0066$ and 0.0003 on days 25 and 29, respectively, and < 0.0001 on days 33–53).

Discussion

In this study, we report construction of the first human Ad secreting a paracrine adaptor molecule. Secretion of sCAR-EGF was demonstrated with low EGFR cells (*Lanes 1 and 2*, Fig. 1), but not with high EGFR HeLa cells (23). In contrast, secretion was detected when HeLa cells were infected with AdsCAR6H, which codes for ectodomain of CAR but not EGF (*Lane 9*, Fig. 1*B*). EGF exhibits high affinity binding to EGFR, which leads to rapid internalization but no recycling of the receptor-ligand complex (12). Thus, perhaps secreted sCAR-EGF also internalizes. Alternatively, binding without internalization would also limit the amount of sCAR-EGF in the supernatant.

Supporting the capacity of sCAR-EGF to mediate binding and subsequent internalization of Ad, supernatant containing the fusion molecule resulted in dose-dependent increases in marker gene expression (Fig. 2). The shape of the curves suggests that the upper limit of retargeting potential was not reached. In an *in vitro* system, it is difficult to assess the capability of a secreted fusion molecule to block fiber-CAR interaction, because in the absence of CAR binding, uptake of Ad into cells can also occur via alternative mechanisms (10). However, we observed up to 52% reduction in luciferase expression with sCAR6H, which could translate into partial blocking of CAR-mediated internalization (into normal cells) by sCAR-EGF *in vivo*, but additional studies are needed.

We used a dual-virus system (CRAdsCAR-EGF) to evaluate the combination of oncolysis and EGFR targeting and saw dramatically increased killing of cells relatively low in CAR but high in EGFR expression, a combination commonly seen with primary cancer cells (Fig. 3). *In vitro*, the isogenic control virus (CRAdCMVLuc) is expected to enter cells even if they are low in CAR (10). The observed difference in oncolysis may result from more rapid internalization of the retargeted virus because of a higher number of receptors. In a living organism, extracellular viruses are at risk for neutralization by immune defenses or being swept away into organs responsible for Ad clearance. Thus, the advantage of rapid binding and internalization could be more pronounced.

s.c. xenografts are a stringent model for testing an oncolytic effect, because viral replication is balanced against rapid tumor growth. Here, we demonstrated significantly improved therapeutic efficacy of CRAdsCAR-EGF in comparison with the isogenic control not secreting sCAR-EGF (Fig. 4).

No signs of sCAR-EGF-causing toxicity were evident when cells were infected with AdsCAR-EGF in comparison with AdsCAR6H. When sCAR-EGF was added daily to SKOV3.ip1 cells infected with a CRAD, no evidence of toxicity to cells was seen. Moreover, obvious signs of toxicity were absent in mice whose xenografts were infected with CRAdsCAR-EGF. Additional studies will show whether the adaptor molecule has an effect of cell growth or whether there is toxicity *in vivo*. Also, it remains to be seen whether sCAR-EGF mediates Ad internalization via the EGFR pathway or merely substitutes for CAR in binding Ad for the native entry mechanism via penton base arginine-glycine-aspartic acid and cellular integrins.

This is the first report of a retargeting molecule secretory from human cells, but this strategy could be feasible with various high-affinity, cancer-specific ligands. Because rapid screening methods allow recognition of large numbers of cancer-specific features, unlimited possibilities for retargeting with secretory-targeting moieties

may soon be available. The dual-virus system used here provides a useful model for rendering AdsCAR-EGF replicative and investigating the combination of oncolysis and retargeting, but efficacy could be improved when *sCAR-EGF* is genetically incorporated into a CRAD.

In conclusion, we show that retargeting of replicating Ad to a receptor overexpressed in cancers is a powerful way of increasing tumor transduction and allows overcoming the lack of the primary Ad receptor. Clinical translation of this approach may be effective in treatment of a variety of human cancers that overexpress EGFR.

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